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(54) Title: SELF-REARRANGING DNA VECTORS

(57) Abstract: Disclosed are replicatable viral DNA vectors encoding a site-specific DNA-altering enzyme and a DNA target recognized by the enzyme, the enzyme selectively converting, in a cell expressing the enzyme, the DNA vector to a rearranged form. The invention further relates to methods for assembling recombinant adenoviral DNAs. These methods include the steps of: providing a first linearized DNA vector including a restriction site and a cos site and a second linearized DNA vector including the restriction site, an adenoviral nucleic molecule, and a cos site; and ligating the first and second linearized DNA vectors, the ligation assembling a recombinant adenoviral DNA.

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SELF-REARRANGING DNA VECTORS

Background of the Invention

The invention relates to DNA vectors.

Mammalian cell expression vectors based on DNA viruses have been widely discussed as gene delivery vehicles for genetic therapy. Among the different DNA viruses proposed for this purpose have been adenoviruses, baculovirus, Epstein Barr virus, and herpes simplex virus. In addition other smaller viruses that have an intranuclear phase in which the viral genome is present as a double stranded DNA, such as retroviruses and parvoviruses, have been proposed as gene delivery vehicles.

Adenoviral vectors (AdV), for example, have a recognized potential for gene delivery, founded in their broad host range, robust growth in culture, and capacity to infect mitotically quiescent cells (Graham and Prevec, Manipulation of adenovirus vectors, p. 109-128, *In* E. J. Murray (ed.), Methods in Molecular Biology, vol. 7, Humana, Clifton, NJ, 1991; Trapnell and Gorziglia, Curr. Opin. Biotechnol. 5:617-625, 1994). AdV can be propagated in a helper cell line, 293, a human embryonic kidney cell line transformed by adenovirus type 5 (Graham et al., J. Gen.Virol. 36:59-72, 1994). 293 cells express the viral E1 gene products (E1a and E1b) that are the master regulatory proteins for subsequent viral gene expression. E1 deleted viruses can propagate in 293 cells, but not in other cells. Although it would be expected that E1 deleted viruses lack the machinery to express viral genes, several studies have demonstrated that cellular E1-like components can stimulate viral gene expression (Imperiale et al., Mol. Cell. Biol. 4:867-74, 1984; Onclercq et al., J. Virol. 62:4533-7,1988; Spergel et al., J. Virol. 66:1021-30, 1992). The expression of these viral genes results in the relatively rapid elimination of transduced cells *in vivo* as a result of cytotoxic T cell responses (Yang et al., Immunity 1:433-42, 1994;. Yang et al., Gene Ther. 3:137-44, 1996; Yang et al., J. Virol. 69:2004-15, 1995).

Thus attention has been focused on eliminating the remaining vestiges of viral expression. Viral genes that have been deleted for this purpose include the gene for E4 proteins (Armentano et al., Hum. Gene Ther. 6:1343-53, 1995; Kochanek et al., Proc.

Natl. Acad. Sci. USA 93:5731-6, 1996; and Yeh et al., J. Virol. 70:559-565, 1996), DNA binding protein (Engelhardt et al., Proc. Natl. Acad. Sci. USA 21:6196-6200, 1994; and Gorziglia et al., J. Virol. 70:4173-8, 1996), DNA polymerase (Amalfitano et al., J. Virol. 72:926-33, 1998), and the preterminal protein (Schaack et al., Proc. Natl. Acad. Sci. USA 93:14686-91, 1996). The most aggressive approach has been the creation of helper virus-dependent vectors that lack all viral genes (Hardy et al., J. Virol. 71:1842-9, 1997; Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-6, 1996; Lieber et al., J. Virol. 70:8944-60, 1996; Mitani et al., Proc. Natl. Acad. Sci. USA 92:3854-8, 1995; and Parks et al., Proc. Natl. Acad. Sci. USA 93:13565-13570, 1996). These vectors have high capacity, evoke reduced cellular immune responses and show prolonged expression *in vivo* (Morsy et al., Proc. Natl. Acad. Sci. USA 95:7866-71, 1998). However to deploy these viruses on the scale required for human clinical application presents major challenges because a cesium chloride (CsCl) gradient is needed to remove the helper virus.

Summary of the Invention

In one aspect, the invention features a replicatable viral DNA vector encoding a site-specific DNA-altering enzyme and a DNA target recognized by said enzyme, said enzyme selectively converting, in a cell expressing said enzyme, said DNA vector to a rearranged form.

In preferred embodiments, the rearranged form includes an autonomously replicating episome and a linear DNA product. In other preferred embodiments, the vector comprises adenoviral DNA.

In yet other preferred embodiments, the vector includes a genetically-engineered recombination site (such as a target of Cre or FLP). Preferably, such a recombination site includes a recognition sequence of a site-specific DNA altering enzyme.

In another preferred embodiment, the site-specific DNA altering enzyme is a recombinase (such as Cre or FLP) or an integrase. Preferably, such an enzyme is functional in a mammalian cell. Preferred embodiments of the vector also include an origin of replication that functions in a mammalian cell (such as an Epstein Barr Virus replicon). Moreover, the vector typically includes a gene of interest (such as a therapeutic gene that encodes a protein or polypeptide or an RNA product).

In another aspect, the invention features a method for assembling a recombinant adenoviral DNA. The method, in general, includes the steps of: (a) providing a first linearized DNA vector comprising a restriction site and a cos site and a second linearized DNA vector comprising the restriction site, an adenoviral nucleic acid molecule, and a cos site; and (b) ligating the first and second linearized DNA vectors, the ligation assembling a recombinant adenoviral DNA.

In preferred embodiments, the first linearized DNA vector comprises a selectable marker (such as a gene encoding a polypeptide that confers, on a host cell expressing such a polypeptide, resistance to an antibiotic). In other preferred embodiments, the first linearized DNA vector includes an adenoviral left-end inverted terminal repeat; a gene of interest; or both. In still other preferred embodiments, the second linearized DNA vector includes a selectable marker. Preferably, the second linearized DNA vector includes an adenoviral right-end inverted terminal repeat.

The method further includes packaging the assembled adenoviral DNA into a phage and infecting a host cell. Typically the first and second linearized DNAs include cosmid vector DNA. In addition, such adenoviral DNA is typically flanked by cleavage sites (such as intron endonuclease cleavage sites).

In another aspect, the invention features an adenovirus producer cell having a nucleic acid molecule that expresses a dominant negative site-specific DNA-altering enzyme. In preferred embodiments, the site-specific DNA altering enzyme is a dominant negative recombinase (for example, a Cre recombinase such as CreY324C or a Flp recombinase). Exemplary adenovirus producer cells include, without limitation, 293 human embryonic kidney cells, per.C6 cells, and N52 cells.

In yet another aspect, the invention features a vector comprising, in the 5' to 3' direction, a first genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme; a gene of interest; a lineage-specific gene promoter; a second genetically engineered *cis*- acting target recognized by a site-specific DNA altering enzyme; and a nucleic acid molecule encoding a site-specific DNA altering enzyme.

In still another aspect, the invention features a vector including, in the 5' to 3' direction, a first genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme; a gene of interest; a bi-directional promoter, comprising a second genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme; and a nucleic acid molecule encoding a site-specific DNA altering enzyme.

In related aspects, the invention features a method of gene therapy including the administration to a patient in need of gene therapy a therapeutically effective amount of the vector of the invention, which is expressed in the patient. The invention further relates to a population of cells transfected with the vector of the invention.

Accordingly, the invention further relates to the use of a recombinant viral vector or use of a recombinant viral particle for gene therapy. Such vectors and viral particles may be introduced either *in vitro* into a host cell removed from the patient, or directly *in vivo,* into the body to be treated, according to standard methods known in the art.

The invention also relates to a pharmaceutical composition that includes a therapeutically effective amount of a recombinant viral vector or viral particle prepared according to the methods disclosed herein, in combination with a vehicle that is acceptable from a pharmaceutical standpoint. Such a pharmaceutical composition may be prepared according to the techniques commonly employed and administered by any known administration route, for example systemically (in particular, by intravenous, intratracheal, intraperitoneal, intramuscular, subcutaneous, intratumoral, or intracranial routes) or by aerosolization or intrapulmonary administration.

One skilled in the art will appreciate that suitable methods of administering a vector (particularly an adenoviral vector) of the present invention to an animal for purposes of gene therapy, chemotherapy, and vaccination are available, and, although more than one route can be used for administration, one particular route may provide a more immediate and more effective reaction than another. Pharmaceutically acceptable excipients also are well known to those who are skilled in the art, and are readily available. The choice of excipient will be determined, in part, by the particular method used to administer the recombinant vector or particle. Accordingly, there are a wide variety of suitable formulations for use in the context of the present invention.

By "recombinant DNA vector" is meant a DNA sequence containing a desired sequence (such as a gene of interest) and an appropriate regulatory element(s) necessary for the expression of the operably linked sequence in a particular host organism (such as a mammal).

By "operably linked" is meant that a gene and a regulatory element(s) are connected to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

By "regulatory element" is meant a genetic element that controls some aspect of the expression of a nucleic acid sequence. For example, a promoter is a regulatory element that facilitates the initiation of transcription of an operably linked coding region. Other genetic regulatory elements include, without limitation, splicing signals, polyadenylation signals, and termination signals. For example, transcriptional regulatory elements in eukaryotes include promoter and enhancer elements. Promoters and enhancers include arrays of DNA sequences that interact directly or indirectly with cellular proteins involved in transcription. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in mammalian cells and viruses.

By "transfection" is meant the introduction of foreign DNA into eukaryotic cells. Transfection is typically accomplished by a variety of means known in the art including, without limitation, calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, and biolistics.

By "stably transfected" is meant the introduction of foreign DNA into the genome of the transfected cell. In general, transfer and expression of transgenes in mammalian cells are now routine practices to those skilled in the art, and have become major tools to carry out gene expression studies and to generate vectors useful in gene therapy.

By "gene of interest" is meant a gene inserted into a vector whose expression is desired in a host cell. Genes of interest include, without limitation, genes having therapeutic value, as well as reporter genes. A variety of such genes are useful in the invention, including genes of interest encoding a protein, which provides a therapeutic function. In addition, the gene of interest, if a therapeutic gene, can render its effect at the level of RNA, for instance, by encoding an antisense message or ribozyme, a protein which affects splicing or 3' processing (e.g., polyadenylation), or it can encode a protein which acts by affecting the level of expression of another gene within the cell (i.e., where gene expression is broadly considered to include all steps from initiation of transcription through production of a processed protein), for example, by mediating an altered rate of mRNA accumulation, an alteration of mRNA transport, and/or a change in post-transcriptional regulation.

By "reporter gene" is meant a gene sequence that encodes a reporter molecule (including an enzyme). A "reporter molecule" is detectable in any detection system, including, but not limited to, enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. Exemplary reporter gene systems include the *E. coli* beta-galactosidase or glucuronidase genes, green fluorescent protein (GFP), blue fluorescent protein (BFP), the human placental alkaline phosphatase gene, the chloramphenicol acetyltransferase (CAT) gene; other reporter genes are known in the art and may be employed as desired.

By "transgene" is meant any piece of DNA, which is inserted by artifice into a cell, and becomes part of the genome of the organism, which develops from that cell. Such a transgene may include a gene that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell that includes a DNA sequence, which is inserted by artifice into a cell and becomes part of the genome of the organism, which develops from that cell.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "derived from" is meant isolated from or having the sequence of a naturally occurring sequence (e.g., a cDNA, genomic DNA, synthetic, or combination thereof).

By "nucleic acid" is meant a polynucleotide (DNA or RNA).

By "gene" is meant any nucleic acid sequence coding for a protein or an RNA molecule.

By "gene product" is meant either an untranslated RNA molecule transcribed from a given gene or coding sequence (such as, mRNA or antisense RNA) or the polypeptide chain translated from the mRNA molecule transcribed from the given gene or coding sequence. Nucleic acids according to the invention can be wholly or partially synthetically made, can comprise genomic or complementary DNA (cDNA) sequences, or can be provided in the form of either DNA or RNA.

The presently claimed invention affords a number of advantages. For example, applicants' gene therapy vehicles particularly those based on recombinant adenoviruses, minimize the propensity of the vectors to activate host immune surveillance, and thereby maximize the persistence for the DNA transduced. The invention therefore facilitates

the development of gene delivery vectors designed to enhance persistence of virally delivered genes and evade the cellular immune response by severing the connection between the sole adenoviral enhancer and the sequences encoding potentially antigenic viral proteins.

As described in more detail below, the mechanism by which this is accomplished differs significantly from any other previous approaches. For example, to reduce the immunogenicity of vectors it is widely acknowledged that some intervention, such as the removal of key genes, or the prevention of their expression in the cells targeted for therapy, is important; however, many related approaches are directed at the host and have generally focused on the selective induction of tolerance to adenoviral antigens, or similar strategies directed at inducing a temporally restricted or antigen-specific compromise of the immune system.

In addition, the poor persistence of transduced DNA appears to be due in part to immunological rejection of transduced cells and to the inability of the viral DNA to replicate, a feature generally inherent in the design of adenoviral vectors, but one which is not associated with applicants' claimed gene therapy vehicles.

Moreover, some contemporary adenoviral vectors are designed to propagate in specific host cells which provide essential replication factors *in trans*. These vectors are typically based on cell lines which express the master regulatory proteins of the E1 complex, which are required for induction of adenoviral DNA replication. In cells expressing E1 genes, the best studied of which is a human embryonic kidney cell line transformed by DNA from human adenovirus 5 (called HEK293, or simply 293), viruses lacking E1 genes propagate well. Such viruses do not propagate on cell lines which do not express E1, and do not generally propagate well in the target cells to which the therapeutic gene is to be delivered. Cells transduced with E1-deleted adenovirus vectors also do not express high levels of viral genes in the absence of E1. However, the weak residual expression that remains in such vectors appears to be sufficient to induce cellular immune responses that contribute to the destruction of the transduced cells.

In addition, the gene therapy vectors claimed herein are hybrid vectors capable of self-rearrangement to form circular and linear DNA products. The linear DNA has a compromised ability to express adenoviral genes, and therefore has a lower immunological profile. And the circular DNA behaves like a mammalian plasmid, encoding the gene of interest and persisting by autonomous replication in the nucleus.

For example, the circularization of an adenoviral vector via the action of Cre recombinase beneficially places a gene of interest (for example, a therapeutic gene) on a self-replicating episome. Vector circularization occurs in a tissue-targeted manner, for example, as a result of the activation of a synthetic liver-specific promoter upstream of the recombinase Cre. Once circularized, the EBV replicon in the episome confers improved persistence on the therapeutic gene as detected by reporter gene expression and direct assay for the presence of vector DNA sequences.

Furthermore, the invention eliminates the requirement for a helper virus, thus avoiding two potential limitations of that system. First, the continuous expression of Cre recombinase may lead to toxicity in host cells, either as a direct consequence of the protein's activity or via its immunogenicity. Second, the Cre helper virus may itself produce antigenic viral proteins that contribute to the immunologic elimination of infected host cells. In contrast, the self-resolving adenovirus/EBV vector system disclosed herein advantageously provides no alternative source of viral proteins, and Cre expression is terminated upon rearrangement.

In addition, the invention described herein provides tools for analyzing the roles of the enhancer in viral gene regulation and virus growth.

The invention also provides a convenient general system for creating recombinant adenoviruses, which increase their attractiveness as gene transduction tools for basic research. The system, for example, employs two conventional plasmid vectors and a λ phage packaging step. The entire recombinant AdV genome is assembled into a single cosmid that is easily amplified in *E.coli*. The use of intron endonuclease recognition sequences flanking the ITRs enhances virus production while simplifying insertion of therapeutic gene sequences into the pLEP shuttle plasmid. The convenience of this vector system has facilitated the construction of over two hundred recombinant viruses to date.

Other embodiments and advantages of the invention will be apparent from the detailed description thereof, and from the claims.

Brief Description of the Drawings

FIGURE 1A is a schematic diagram of the structure of an adenoviral type A vector and its fate in a target cell. enh refers to the Ad2 enhancer; GFP refers to the marker gene green fluorescent protein; EBV refers to the Epstein Barr Virus replicon;

$TetO_7$ refers to a heptamer of Tet operator; TetR refers to the Tet repressor; VP16 refers to the viral protein 16 of Herpes simplex virus, SD refers to the splice donor site; and SA refers to the splice acceptor site.

FIGURE 1B is a schematic diagram of the structure of an adenoviral type B vector and its fate in a target cell. enh refers to the Ad2 enhancer; GFP refers to the marker gene green fluorescent protein; EBV refers to the Epstein Barr Virus replicon; SD refers to the splice donor site; and SA refers to the splice acceptor site.

FIGURE 2A shows a schematic diagram of the pLEP cosmid polylinker region and its position relative to the adenoviral left ITR. The adenovirus enhancer/packaging sequence (ψ) is boxed.

FIGURE 2B is a schematic diagram showing the generation of a single cosmid encoding the AdV genome by the direct ligation of two smaller plasmids. A gene expression unit, CMVGFP, was inserted into the pLEP cosmid at the polylinker region. pLEP and pREP cosmids were digested with an intron endonuclease (PI-PspI), ligated, and packaged *in vitro* to generate pAd2CMVGFP. This DNA was then digested with another intron endonuclease (I-CeuI) to expose the ITRs at both ends of the viral genome. Finally, cosmid digestion mixtures were transfected into 293 cells. Plaques generated by recombinant viruses are detected in 7-10 days.

FIGURE 3A shows the restriction analysis of cosmids carrying the full length AdV DNA showing uniform generation of the desired vector DNA. 2 μg DNA samples from four pAd2-7CMVGFP colonies were digested with Bgl II, resolved on a 1% agarose gel and stained with ethidium bromide. The predicted sizes of the DNA fragments are: 13261, 7684, 5228, 5088, 2284, 1757, 1549, 1270, 351, and 275 base pairs (bp). The 5228 and 5088 fragments appear as a doublet, and the 351 and 275 bp fragments are too small to be seen on the gel.

FIGURE 3B shows the release of the recombinant Ad DNA from cosmids by I-CeuI digestion. 2 μg of pAd2-7CMV DNA from two clones was digested with I-CeuI. Arrows indicated the position of the released recombinant AdV DNA and the vector fragments of approximately 35 kb and 5 kb, respectively.

FIGURE 4A shows the appearance of plaques in 293 cells transfected with 10 μg of pIAdGFPB with no ITR exposed (undigested), one ITR exposed (BsaBI or I-CeuI), or both ITRs exposed (BsaBI plus I-CeuI). Values represent the mean plaque counts per

dish and the time required for plaque development in 293 cells from three separate experiments. "I" designates I-CeuI; and "B" designates Bsa BI.

FIGURE 4B shows the viral titers obtained from plaques that were allowed to grow over 10 days after transfection. Viruses were harvested and the titer of each virus stock was determined by a GFP based semi-quantitative titration procedure described herein. Values represent the mean ± SE of three independent determinations.

FIGURE 5 is a schematic diagram showing a linear AdV that resolves into a circular episome. The elements involved in the self-directed rearrangement of the vector are shown schematically in pLEP1BHCRGFP/EBV and in the corresponding AdV. Starting from the left ITR, the elements are shown as following sequence: left ITR, 147 bp; first 34 bp loxP site; 185 bp enhancer/packaging signal; 64 bp splicing acceptor (SA) from EF1α gene first intron; 720 bp GFP cDNA; 230 bp SV40 poly(A); 1.7 kb TK-EBNA-1/OriP; 970 bp HCR12 promoter; 1 kb EF1α gene first intron containing splicing donor (SD) and acceptor (SA) sites with the second loxP site inserted at 64 bp upstream of the 3'end; 1.2 kb Cre gene tagged with AU1 and a nuclear localization signal; ~120bp poly(A) signal and PI-PspI site. After infection of liver cells, the HCR12 promoter drives the expression of Cre which results in the cleavage of the two loxP sites. This results in the circularization of the fragment containing the EBV replicon. The excision severs the connection between the enhancer/packaging signals and the remainder of the AdV genome. The Cre gene becomes promoterless and is left on the AdV genome fragment. After excision, the HCR12 promoter drives the expression of the GFP reporter gene. The EBV replicon maintains the excised circle as an episome in host cells.

FIGURE 6A is a schematic representation of the loxP sites and EBNA-1 locations in the AdV genome. The relevant Bgl II site is also shown.

FIGURE 6B shows the time course of rearrangement in HepG2 and Hela cells at an equal multiplicity of infection (moi) of 1,000 particles per cell. Cells were infected with Ad2HCRGFP/EBV viruses for 2 hours at 37 °C. Hirt DNA samples were extracted from the cells. ~5 μg of Hirt DNA samples were digested with Bgl II, fractionated on a 1% agarose gel, and analyzed by Southern blot techniques using a ^{32}P-labeled EBNA-1 fragment as the hybridization probe.

FIGURE 6C shows the DNA blot results obtained from Hela cells infected at a moi of 10,000; and HepG2 at 1,000. The upper bands (4915 bp) represent the circularized DNA fragments whereas the lower bands (3162 bp) represent the non-circularized AdV.

FIGURE 7A shows green fluorescent protein (GFP) expression in liver and non-liver cells infected with the Ad2HCRGFP/EBV viruses. Cells were cultured in 35 mm dishes and infected with the Ad2HCRGFP/EBV virus at desired moi. HepG2 cells were infected with 1,000 particles per cell, whereas Hela cells were infected with a moi of 10,000. GFP expression was examined at the indicated time points after infection. Fluorescent cells were photographed using an Olympus SC35mm camera mounted on an Olympus IX70 fluorescent microscope, at 200x magnification, using a filter with peak excitation and emission wavelengths of 450 nm and 510 nm, respectively.

FIGURE 7B shows the expression of GFP in HepG2, Hela, A431, and HT29 cells. Cells were seeded in 35 mm dishes and infected with the Ad2HCRGFP/EBV virus at a moi of 10,000 particles per cell. GFP expression was examined at 72 hours after infection.

FIGURE 7C shows the expression of GFP in human primary hepatocytes. These cells were photographed under bright field (left) and fluorescent conditions (right).

FIGURE 8A shows the results of RT-PCR that was performed to detect the tripartite leader sequence (upper panel) for virus late gene expression; and PCR was performed in the DNA samples for detection of the AdV genomes. The specific target sequences are described in detail *infra*. PCR analyses of adenovirus late gene expression in cells infected with the first generation AdVs or the self-resolving Ad2HCRGFP/EBV was analyzed. HepG2 cells were cultured in 35 mm dishes and infected with increasing moi (0, 10, 100, 1000, 10,000, and 100,000) of adenoviral vectors. RNA and DNA were isolated in parallel from the cells at 72 hours after infection.

FIGURE 8B shows a summary of quantitative RT-PCR and PCR results. Each determinant was the average of three experiments.

FIGURE 9A is a schematic diagram depicting the deletion analysis of the OriP and EBNA-1 regions of the EBV replicon. Structures of the deletions in EBNA-1 and OriP are schematically represented. Elements considered important for episomal maintenance are indicated. FR refers to the family of repeats; DS designates the region

of dyad symmetry; LR1 refers to the so-called linker region 1; GA refers to gly-ala repeats; LR2 refers to linker region 2; and Dimerization designates the dimerization domain.

FIGURE 9B is a graph depicting fractions of GFP positive cells carrying the EBV replicons represented in Figure 9A.

FIGURE 10A shows the positions and identities of Cre mutants tested for their dominant negative Cre activities.

FIGURE 10B is a schematic diagram of the substrate Cre plasmid (ad2239) used to test dominant negative functions of Cre mutants.

FIGURE 10C shows GFP expression in cells cotransfected with the substrate Cre plasmid (ad2239) and the indicated Cre mutants.

FIGURES 10D and 10E show Cre mutants tested for their ability to inhibit rearrangement. Only those showing the strongest inhibitory activities were retested in Fig. 10E. GFP intensity was normalized to that of cells in the absence of inhibition.

FIGURE 11 shows GFP expression in 293 TetON cells and #17 cells transfected with ad2239. The ability of #17 cells to inhibit Cre activity is demonstrated by the weak GFP signal in cells treated with 2 μM doxycycline.

FIGURE 12 is a schematic diagram depicting the tetracycline mediated auto-regulatory circuit.

FIGURES 13A and 13B show the effects of different basal elements on synthetic TetO promoter activity. FIGURE 13A shows a schematic diagram of the components of various auto-regulatory synthetic TetO promoters. FIGURE 13B shows a comparison of the strength of auto-regulatory synthetic TetO promoters bearing different basal elements, in the presence and absence of tetracycline, using GFP as a marker in HepG2 cells.

FIGURE 14 shows the structure of a Cre substrate plasmid (ad2265). The promoter, Ef1α, and the gene, BFP, are interrupted by two loxP sites, which can be joined by Cre-mediated recombination. PA stands for poly A; BFP for blue fluorescent protein.

FIGURES 15A and 15B show the estrogen regulation of Cre recombinase activity. 293 cells infected with type B virus, AD121.5, in which the Cre enzyme is fused with estrogen ligand binding domain at both the N- and C-termini were cultured in the presence or absence of 1 μM estrogen. Cre-mediated rearrangement in the presence

of estrogen is shown in Figure 15A, whereas blot analysis of extrachromosomal DNA from the same cells is shown in Figure 15B. L represents the position corresponding to the unrearranged adenoviral DNA; and C represents the position corresponding to the circular form of DNA.

FIGURE 16 shows the rearrangement of adenoviral sequences *in vivo*. Extrachromosomal DNA from the livers of Rag-2 mice sacrificed 2.5 hrs post injection of type A adenovirus, AD102.7, was analyzed by DNA blot. L represents the size corresponding to linear adenoviral DNA; and C represents the size corresponding to rearranged circular DNA.

FIGURE 17 is a photomicrograph depicting high level GFP expression in Rag2 mouse hepatic tissues 48 hrs post type A adenovirus (AD102.7) injection.

FIGURE 18A and 18B show schematic diagrams of the structures of adenoviral vectors and their fates in target cells. enh refers to Ad2 enhancer; GFP refers to green fluorescent protein; EBV refers to Epstein Barr Virus replicon; $TetO_7$ refers to heptamer of Tet operator; TetR refers to Tet repressor; VP16 refers to transcriptional activator domain from HSV protein 16; SD refers to splice donor site; and SA refers to splice acceptor site.

FIGURE 19A shows the structure of a FLP substrate plasmid, ad2879. The promoter, Ef1α, and the gene, GFP, are interrupted by 2 FRT sites, which can be joined by the FLP-mediated recombination. PA stands for poly A; BFP for blue fluorescent protein.

FIGURE 19B shows the structure of a cre substrate plasmid, ad2204.

FIGURE 20 shows the structures of several FLPe anti-sense plasmids.

FIGURE 21 is a panel of photomicrographs showing inhibition of FLP enzyme activity by anti-sense FLP. 293 cells were transfected with FLP substrate (Figure 12) and plasmids indicated in each photo. High GFP intensity indicate the higher expression of FLP and less inhibition by the anti-sense expressed.

FIGURE 22 shows a schematic diagram of FRT/Cre and loxP/FLP adenovirus.

Detailed Description

Described herein are systems for the regulated self-rearrangement of DNA vectors, for example, gene therapy vectors. Such regulated self-rearrangement has the potential to prevent unwanted expression of vector genes not required for a therapeutic effect, and to allow the stable association of the therapeutic gene with the target cell.

The essential elements of the regulated DNA rearrangement system are a gene which encodes one or more proteins which induce DNA rearrangement, a method for regulating the activity of those proteins or their abundance, and a target DNA sequence on which those proteins act. Particularly desirable are methods for regulating the activity of the proteins or their abundance which can be easily carried out on an intact organism, such as administration or withdrawal of a drug, hormone, or environmental stimulus such as heat or irradiation, which induces the activity or abundance of the proteins which cause DNA rearrangement.

Especially desirable are regulated DNA rearrangement systems in which all of the components can be delivered in a single vector. An example of this is a virus which bears both the cis-acting sequences for DNA rearrangement as well as the protein or proteins which act on those sequences, and the regulatory apparatus which controls the activity or abundance of those proteins. However, it is not necessary that the different elements be encoded in a single nucleic acid.

The important elements of this strategy are: the compromise of vector gene function by regulated rearrangement of DNA topology, the generation of plasmid circles from vector DNA in a regulated manner, and the removal of enhancer or promoter elements from the vector DNA by regulated excision. It is also important that the circular DNA generated by site-specific recombination possesses a mechanism for stable association with the host genome in some form, here conferred by the EBV replicon. In other embodiments, the circular DNA might possess the ability to direct its integration into the host chromosomes by a site-specific integration. Site-specific integration into the host chromosomes may also be generated by the action of a regulated site-specific recombinase on a linear template without passing through a circular intermediate.

Also described herein is one particular self-rearranging vector that begins as a hybrid adenovirus vector which is capable of converting itself into two unlinked molecules, a circular and a linear DNA. After this event the linear DNA product is deleted for two important cis-acting sequences: the packaging signals, which are

required for insertion of the viral DNA into the viral capsid, and the enhancer, which increases the expression of other promoters encoded in the viral DNA. The remaining linear DNA is thereby compromised in its ability to express adenoviral genes, endowing the vector with a lower immunological profile. The circular DNA generated by the excision event is a mammalian cell plasmid which has the capacity to persist by autonomous replication in the nucleus. This capacity is encoded in genetic elements derived from the Epstein Barr virus (EBV). A schematic diagram of such a vector is illustrated in Figure 5.

Epstein Barr virus is a human herpes virus which is the etiologic agent of infectious mononucleosis and which has been implicated in the genesis of Burkitt's lymphoma, a B cell neoplasm, and is thought to be a predisposing factor for some forms of nasopharyngeal carcinoma. Approximately 85% of the adult Western population has a persistent population of B cells which contain a circular latent form of the viral-genome, maintained in cells by the action of Epstein Barr Nuclear Antigen 1 (EBNA1), a DNA replication protein that acts on the viral latent phase origin of replication, OriP. EBNA1 in and of itself is not thought to promote neoplasia; current thinking places greater weight on the actions of the EBNA2 proteins and LMP, latent membrane protein, in the inception of EBV-associated neoplasm.

Mammalian cell plasmids have been created which bear the EBNA1 gene and OriP. In nonrodent cells, these plasmids persist by replication with each transit of the cell cycle. Multiple transcription units can be borne by these plasmids, allowing regulated expression of diverse gene products.

Preferred adenoviral vectors, shown in Figures 1A and 1B, are linear forms of an EBV plasmid flanked by loxP sites, cis-acting sequences required for site-specific recombination directed by the bacteriophage P1 cre protein. To prepare an adenovirus bearing both the cre protein and loxP sites, it is necessary to insure that the cre protein is not expressed while the vector is being propagated in 293 cells. To lower the immunological profile of the vector, it is also desirable that the cre protein not be expressed after the vector delivered its payload to the target cell and the cre protein performed its function.

To accomplish these objectives, two general approaches have been developed for the production of adenoviral chromosomes that circularize following the regulated expression of site-specific recombinases. In each case, the vector is engineered to allow

for the production of viruses in 293 cells, and to provide transitory expression of recombinase that induces rearrangement in target tissues. The major difference between the two strategies lies in the way the deinduction of recombinase is achieved.

In the first approach, adenoviral vectors are engineered to turn an activating transcription factor into a repressor upon chromosomal rearrangement. Vectors employing this approach are referred to herein as type A vectors (Figure 1A). In the second approach, the recombinase promoter is redirected following chromosomal rearrangement. Vectors utilizing the second approach are referred to as type B vectors (Figure 1B). In both cases a linear chromosome is converted to its circular episomal form and a resulting deleted linear form. The circular DNA contains an Epstein Barr virus (EBV) replicon, which allows synchronous replication of the episome with the host mitotic cycle (Reisman et al., Mol. Cell Biol. 8: 1822-32, 1985; Yates et al., Nature 313: 812-15, 1985). The linear DNA is deleted for the enhancer and E1 genes.

One self-regulated gene switch, employing the type A vector strategy, was designed based on the bacterial transposon Tn10 tetracycline repressor (tetR) gene. In its natural context, the tetR protein binds to specific sequences (tet operator sequences) upstream of a tetracycline resistance gene, preventing transcription of the gene unless tetracycline is present. To adapt this protein for eukaryotic gene regulation, a gene fusion is created between tetR and an active portion of a strong eukaryotic transcriptional activator, the herpes simplex virus VP16 protein. The fusion protein exerts its action on a synthetic promoter created by the insertion of multiple tet operator sequences upstream of a basal promoter element. This configuration allows high-level gene expression whenever the tetR-VP16 fusion protein binds to its cognate operator sequences. Because the tetR protein normally does not bind to its operator in the presence of tetracycline, the activity of this synthetic promoter is high in the absence of tetracycline and low in its presence.

One example of a type A vector is shown in Figure 1A. This self-regulated gene expression cassette, present in a hybrid adenovirus, consists of a bi-directional promoter element in which central tetR binding sites are flanked by divergently oriented basal promoter elements. In one direction the promoter directs the formation of a transcript encoding the cre protein; in the other direction, the promoter directs the formation of a tetR-VP16 fusion protein. The latter differs from the conventional version in bearing a loxP site between the tetR component and the VP16 component. When tetracycline is

present this gene switch is silent. As shown in Figure 1A, upon introduction into a target cell in the absence of tetracycline, the tetR-loxP-VP16 fusion protein is produced, stimulating further production of the fusion protein, and the cre protein. The cre protein then acts to promote site specific recombination between the loxP site in the tetR-loxP-VP16 coding sequences, and a distant loxP site. As a result of this recombination, the fusion protein coding sequence is disrupted so that the promoter no longer directs the formation of a tetR-loxP-VP16 fusion protein, but gives rise to an inert tetR-loxP-VP16 fusion protein for binding to the promoter upstream elements, thereby extinguishing promoter activity.

As shown in Figure 1A, the excised circular DNA element contains at least two transcription units. In addition, other transcription units or internal ribosome entry site elements may be used to allow the coexpression of gene products which are useful for extending the persistence of the delivered DNA, regulating expression of the gene of interest, or providing for ablation of the transduced cells once their presence is no longer desirable. In addition, the linear DNA remaining after excision of the circular gene expression plasmid lacks both viral packaging sequences and the cis-acting enhancer. Within this linear DNA, additional loxP sites may be placed to provide for the rearrangement of the remaining vector DNA in the target cell, disrupting the normal topology of the genes, and further thwarting expression.

Using the type B vector design strategy, described in greater detail below, a recombinant adenoviral gene delivery system that is capable of undergoing growth phase-dependent site-specific recombination has also been constructed.

The following examples are presented for the purpose of illustrating, not limiting, the invention.

TYPE B VECTORS – EXPERIMENTAL RESULTS

Several experimental examples for constructing type B vectors and for carrying out the general approaches of the invention are now described below.

Two-Cosmid System for Efficient Construction of Recombinant AdV

To simplify and facilitate the generation of recombinant AdV, a system was established to assemble the desired AdV genome in a single plasmid by ligation (shown in Figures 2A, 2B). The system consists of two component vectors, a left end plasmid,

pLEP, and a right end plasmid, pREP. The left end Ad sequences (nt 1-376) in pLEP include the viral inverted terminal repeat, the cis-acting packaging sequences, and the viral enhancer. Nucleotide (nt) positions described herein refer to the wild type Ad2 sequence in GenBank (J019017). The Ad sequences are followed by the gene expression unit intended for delivery and an intron endonuclease (PI-PspI) cleavage site. The right end plasmid contains a PI-PspI site followed by the Ad2 genome from the end of the E1 locus rightward (nt 3527-35937).

pLEP is a small tractable vector for cloning, whereas pREP is much larger and contains less frequently manipulated genes. Both pLEP and pREP contain a bacteriophage λ *cos* site, oriented to generate a single cosmid of appropriate length for *in vitro* packaging following ligation of the two plasmids at the PI-PspI cleavage site. pLEP is tetracycline resistant (Tet^r) and pREP is ampicillin (Amp^r) resistant, allowing the recombinants to be selectively isolated by co-selection for both markers. In the resulting assembled cosmid, the adenoviral sequences are closely flanked by cleavage sites for the intron endonuclease I-CeuI. Digestion with I-CeuI liberates the entire recombinant AdV genome from the parent cosmid (see Figure 2B).

Three classes of pREP have been constructed to allow the preparation of AdVs bearing E1 (pREP7; SEQ ID NO.: 2), E1 and E3 (pREP8; SEQ ID NO.: 3), or E1, E3, and E4 (pREP12; SEQ ID NO.: 4) deletions. pREP7 (SEQ ID NO.: 2) contains nt 3527-35937 of the Ad2 genome, and pREP8 (SEQ ID NO.: 3) carries an additional deletion in the E3 region (Δ nt 27901-30841). pREP12 (SEQ ID NO.: 4) has deleted open reading frames (ORF) 1-4 of the E4 region (Δ nt 34121-35469, 1348 bp). AdV generated with these cosmids should be able to accommodate 5, 8, and 10 kb inserts, respectively.

These aforementioned vectors were constructed as follows. The EcoRI to BsaI fragment that spans the ampicillin resistance gene in pBR322 was deleted and replaced by a synthetic adapter, and the bacteriophage λ cos site was inserted between the unique StyI and BsmI sites. A PCR amplified Ad2 fragment containing the left end ITR (L.ITR), enhancer elements, and the encapsidation signal (nt 1-376) was created and inserted into the adapter (Figures 2A, 2B) to yield the tetracycline-resistant left-end plasmid pLEP. The right end of Ad2 from the AflII site to the right end (nt 3527-35937) was assembled into an ampicillin resistant cosmid vector, pACKrr3 (SEQ ID NO.: 1), by multiple steps of PCR amplification and fragment interchange. The resultant cosmid

was termed pREP7 (SEQ ID NO.: 2). To expand vector capacity, two deletions were incorporated into the pREP7 (SEQ ID NO.: 2) cosmid, an E3 gene deletion (nt 27901-30841, 2840 bp); cosmid pREP8 (SEQ ID NO.: 3) and a 1.3 kb deletion (nt 34121-35469) in the E4 region of the Ad2 region; pREP12 (SEQ ID NO.: 4)

An example of the construction of an AdV carrying a CMV-GFP expression unit is outlined in Figure 2. pLEPCMVGFP (Tet^r) was digested with PI-PspI and ligated to the pREP7 (SEQ ID NO.: 2; ΔE1, Amp^r) digested with the same enzyme. The ligation mixture was packaged with λ phage extracts (MaxPlax lambda packaging extracts, Epicentre Technologies) and a fraction of the packaged phage was used to infect a recombination-deficient *E. coli* host, with selection for the assembled plasmid on Amp/Tet plates. Transductants containing pLEP fused to pREP were selected on agar containing 25 μg/ml ampicillin and 12.5 μg/ml tetracycline (Amp/Tet). Colonies were selected and DNA isolated (Qiagen). DNA was used either for restriction analysis or for tranfection of 293 cells as described herein.

Figure 3A shows typical results for the Bgl II digestion pattern of a pLEP3CMVGFP/pREP7 hybrid cosmid, pAd2-7CMVGFP DNA. Because of the size minimum (~40 kbp) for λ phage *in vitro* packaging and the double antibiotic selection, most of the colonies growing on Amp/Tet plates were the desired hybrid cosmids, and undesired rearrangements were rarely seen. In the present example, all four pAd2-7CMVGFP clones exhibited the digestion pattern predicted from the inferred sequence. The entire recombinant AdV genome was then released from the cosmid by I-CeuI digestion (Figure 3B). I-CeuI digestion leaves ten nucleotides to the left of the left ITR and eight nucleotides to the right of the right ITR. Short flanking sequences have been reported to be eliminated during replication of recombinant viruses after transfecting the DNA into 293 (human embryonic kidney) cells (Hanahan et al., Mol. Cell. Biol. 4:302-309, 1984).

The digestion reaction can be transfected into 293 cells without purification as follows. 293 cells, obtained from Microbix Bisosystems (Ontario, Canada), were cultured in 10 cm dishes in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2mM glutamine and penicillin/streptomycin (Gibco BRL), and maintained at 37 °C and 5% CO_2 atmosphere incubator. Cells were grown to ~50% confluence on the day of transfection. Ten μg of cosmid DNA were digested with I-CeuI in a volume of 50 μl. The reaction mixture was transfected into 293 cells by

calcium phosphate precipitation (Graham and Prevec, Manipulation of adenovirus vectors, p. 109-128, *In* E. J. Murray (ed.), Methods in Molecular Biology, vol. 7, Humana, Clifton, NJ, 1991) without purification. After transfection, cells were cultured and examined daily for the appearance of cytopathic effects (CPE). Virus propagation, purification, plaque assay, and viral DNA isolation were performed using established protocols (Graham and Prevec, *supra*). At day six post-transfection, 5-30 viral plaques/10 cm dish /10 μg DNA were usually apparent, which compared favorably with the 30-50 plaques/10 cm dish /10 μg DNA found for 293 cells transfected with purified wild type Ad2 DNA.

To compare the efficiency of recombinant virus production, similar viruses were also generated by homologous recombination. 20 μg of pREP7 (SEQ ID NO.: 2) was co-transfected into 293 cells with 10 μg of a plasmid encoding the left end of the adenoviral genome and a green fluorescent reporter gene (pLITREF1αGFP). pLITREF1αGFP contained the Ad2 left end nt 1-376, an EF1α promoter/GFP expression unit and Ad2 sequence (from 3525-8120) that overlaps with the same sequence in pREP7 (SEQ ID NO.: 2). This overlap fragment served as the region for homologous recombination. Each co-transfection was performed in duplicate. Initial plaques took longer to appear (14 days post transfection) and were less abundant (0-3 plaques per plate).

Data in the literature suggest that exposed ITR ends favor efficient virus production (Hanahan et al., *supra*). To assess the importance of this effect, an AdV cosmid, pIAdEF1αGFPB, in which the AdV ITRs were flanked with a different restriction site at each end was constructed. pIAdEF1αGFPB DNA was digested with BsaBI to expose the right ITR, I-CeuI to expose the left ITR, or the two enzymes were used together to expose both ends. Digested cosmid DNA samples were transfected into 293 cells and plaques were allowed to develop. Virus propagation, purification, plaque assay, and viral DNA isolation were performed using established protocols described in Graham and Prevec. (Manipulation of adenovirus vectors, *In* E. J. Murray (ed.), Methods in Molecular Biology, vol. 7. Humana, Clifton, NJ., pp. 109-128, 1991).

Ten days after transfection the viruses were harvested and viral titers were determined. The average titer for the viral stocks (Figures 4A, 4B) was 1.3×10^4 pfu/ml from transfection with undigested DNA; 2.4×10^5 pfu/ml from BsaBI linearized DNA (free right ITR); 1.1×10^5 pfu/ml from I-CeuI linearized DNA (free left ITR); and 2.7 x

10^6 pfu/ml for the BsaBI/I-CeuI double digested DNA (both ITRs free). Thus liberation of each end resulted in an approximate increase in the efficiency of generating virus by a factor of ten (Figures 4A, 4B).

Construction of an AdV Capable of Self-Rearrangement

One approach to attenuating adenoviral gene expression and improving transgene persistence is the creation of viruses capable of undergoing internal, self-directed rearrangement upon delivery to the target tissue. In principle, this objective can be achieved through the regulated expression of site-specific recombinases in vectors that contain the cis-acting target of recombinase action. To allow such vectors to be created, the recombinase activity must be suppressed during propagation in the packaging cell line. As described in more detail below, the use of a lineage-specific promoter to control recombinase expression has been successfully employed to achieve this end.

An example of this is shown in Figure 5. The expression of Cre recombinase was controlled by a liver-specific promoter constructed as follows. The human hepatic control region 1 and 2 (HCR1 and 2) of the ApoE/C gene locus (Allan et al., J. Biol. Chem. 270:26278-81, 1995; and Dang et al., J. Biol. Chem. 270:22577-85, 1995) were amplified by PCR using 293 cell genomic DNA as the template. The following primers were used to amplify both HCR1 and HCR2 fragment: HCRtop-5'gcggaattcggcttggtgacttagagaacagag 3' (SEQ ID NO.:5); HCRbot – 5' gcgggatccttgaacccggaccctctcacacta 3' (SEQ ID NO.:6). The amplified PCR fragments (~0.39 kb) were cloned into pUC19. The HCR1 and HCR2 sequences were confirmed by dideoxy DNA sequencing. The two fragments were assembled in a head to tail orientation, fused with a synthetic basal TATA element and cloned in a parental pLEP vector containing a GFP reporter gene. The resultant plasmid was named pLEPHCR12GFP. The synthetic liver-specific, as demonstrated below, provided a means to control Cre recombinase expression during propagation of the vector in 293 cells, and allowed for testing the consequences of abstracting the enhancer from the linear vector DNA upon delivery of the DNA to the target cells.

In 293 cells, this promoter is silent, allowing the viral chromosome to be propagated with minimal rearrangement. Any rearranged viruses that are formed lack packaging signals and so disappear from the pool of propagating vectors. In liver cells the Cre recombinase is induced by the action of the tissue-specific promoter. The

resulting Cre-induced recombination excises a circular episome and redirects the transcriptional output of the liver-specific promoter so that it directs the synthesis of the transgene of interest. The remaining linear fragment consists of an adenoviral genome lacking the enhancer and packaging signals and a Cre expression unit devoid of promoter sequences.

In the form discussed here, one loxP site is located at nucleotide 147 of the Ad2 genome, between the left ITR and the enhancer/packaging sequences, and the second loxP site is placed inside an intron a few bases upstream of the splice acceptor sequence. Hence the loxP site does not appear in the resulting mature transcript. The Cre coding sequence that remains on the right end linear fragment after rearrangement lies downstream from a splice acceptor that lacks a splice donor or upstream promoter sequences. This effectively terminates the expression of Cre following excision.

Prior to recombination, the Cre recombinase gene is under the control of a synthetic promoter (referred to as HCR12), consisting of hepatic locus control elements from the human ApoE/C locus fused to the first intron of the human EF1α gene. After cyclization the HCR12 promoter lies upstream of the transgene (in this case GFP) and the distal segment of the intron (beyond the loxP site) contains the adenoviral enhancer. To facilitate manipulation of the plasmids in *E. coli*, the human IgG1 hinge-CH2 intron (118 bp) was inserted in the Cre coding sequence at nucleotide 237, suppressing Cre expression in bacteria. The circularized episome contains the latent origin of replication (OriP) and trans-acting DNA replication protein (EBNA-1) of Epstein Barr virus, and hence is capable of autonomous replication in synchrony with the host mitotic cycle (Yates et al., Nature 313:812-815, 1985).

Using the two cosmid system described above, the pLEP plasmid containing the self-resolving components, pLEP1BHCR12, was ligated with pREP8 (SEQ ID NO.: 3; ΔE1ΔE3) to create pAdVHCRGFP/EBV. The latter was digested with I-CeuI and transfected into 293 cells. Appearance of plaques from AdVHCRGFP/EBV was retarded (by 8 days) compared to non-rearranging viruses, perhaps as a result of basal expression of the liver-specific promoter in 293 cells. However high titer viral stocks of 10^{12} nominal (absorbance-determined) particles/ml was achieved.

Rearrangement in Target and Nontarget Cells

To test excision efficiency, HepG2 (hepatocellular carcinoma) and Hela (cervical carcinoma) cells, obtained from ATCC, were infected with virus at a multiplicity of infection (moi) of 1,000 nominal particles/cell. This titer corresponds to approximately 10 plaque forming units per cell. For these experiments, HepG2 and Hela cells were seeded in 35mm dishes and cultured to approximately 80% confluence in DMEM/FBS as described herein. Cells were infected with the desired multiplicity of virus in a volume of 1 ml at 37 °C for 2 hours. At the end of the incubation, cells were washed with PBS twice and cultured in 2 ml of medium. Cells were collected in parallel at desired points for low molecular weight DNA and RNA extraction. Cells were examined for GFP expression by fluorescence microscopy (Olympus, IX70) or microtiter plate reader (PerSeptive Biosystem, CytoFluor II) before extraction of DNA for analysis of chromosomal rearrangement.

DNA analysis of chromosomal rearrangement was performed as follows. 5 μg of Hirt DNA was digested with Bgl II and analyzed by DNA blot techniques using a labeled EBNA-1 gene fragment as probe (Figure 6). The Bgl II fragment from the non-circularized AdV is 3162 bp, generated from the 5'end of the AdV to the first BglII site in the AdV. The circularized fragment created from the two loxP sites has a size of 4915 bp (Figure 6A). Densitometry revealed that at 72 hours post infection, 95% or more of the input genomes had undergone circularization in HepG2 cells. In contrast, low but detectable levels of circularized fragment was visualized in Hela cells infected at the same time and at the same multiplicity of infection used for the HepG2 cells (Figure 6B).

At the time of infection (t=0, Figure 6B), the amount of input viral DNA detected by DNA blot was higher for HepG2 cells than for Hela cells when similar virus multiplicities were applied (moi of 1,000). This may reflect differences in AdV adsorption or infection efficiency between the two cell types, possibly as a result of the lower levels of coxsackievirus-adenovirus receptor on the Hela cells surface. To achieve similar viral genome input into HepG2 and Hela cells, Hela cells were infected with ten-fold more virus (moi of ~10,000) than HepG2 cells (moi of 1,000). Episomal DNA samples were extracted and analyzed by blotting. The results (Figure 6C) indicated that when comparable amounts of viral genome are present in the nucleus, the cyclization rate in both cell types was similar. Because the level of subsequent GFP expression is

much higher in HepG2 cells than in HeLa cells (Figure 7A), it is likely that very small amounts of Cre recombinase suffice to promote rearrangement, and that recombinase expression is not limiting for rearrangement in either HepG2 or HeLa cells.

GFP expression cannot be detected until rearrangement has taken place, so the measurement of the fraction of GFP positive cells provided a simple alternate method for assessing the degree of productive rearrangement. Figure 7A shows that GFP expression developed quickly in transduced HepG2 cells, but that only a few GFP positive cells can be detected in Hela cells infected with a ten fold higher moi, conditions that allow circularization to a comparable extent to that seen in HepG2 cells (Figure 6C).

The HCR12 promoter specificity was also tested by infecting two additional non-hepatic cell lines, A431 (human epidermoid carcinoma) and HT29 (human colon adenocarcinoma), with the Ad2HCRGFP/EBV vector. Both cell lines were obtained from ATCC and cultured using DMEM/FBS as described herein. A few cells, with weak GFP signal, were detected at 72 hours after infection in these cells (Figure 7B). In contrast, these non-hepatic cells could be infected efficiently with a first generation AdV, Ad2CMVGFP virus (data not shown), indicating that the low GFP signal was not due to the low infectivity of these cells by AdV.

To further assess the utility of the AdV genome rearrangement, primary human hepatocytes were infected with the Ad2HCRGFP/EBV vector. For these experiments, primary human hepatocytes, generously provided by Dr. Albert Edge (Diacrin, Inc., Charlestown, MA) were isolated and cultured as described by Gunsalus et al. (Nat. Med. 3:48-53, 1997), infected with adenovirus, and GFP expression was analyzed. As shown in Figure 7C, GFP expression was readily detected 72 hours after infection.

Diminished Viral Gene Expression in Rearranged AdV

After excision, the adenovirus major enhancer/packaging signal segregates with the episomal DNA, yielding a linear fragment containing the remainder of the AdV genome without this important cis-element (Figure 5). To assess the impact of enhancer deletion, PCR amplification and quantitative RT-PCR measurement of late viral gene expression was performed as follows.

Four μg of total RNA was reverse transcribed into cDNA using M-MLV RT by a standard protocol (Promega). 1 μl of the cDNA from each sample was used in subsequent PCR reactions. PCR primers were designed to amplify the tripartite leader sequence of the adenovirus late genes: TPL1 - 5' act ctc ttc cgc atc gct gt 3' (SEQ ID NO.: 7) and TPL2 - 5' ctt gcg act gtg act ggt tag 3' (SEQ ID NO.:8). For detection of the AdV genome in the Hirt DNA samples, 1 μg DNA was employed in the PCR amplification using the following primers which are specific for the adenovirus DNA in the fiber gene: Fiber1 - 5' ccg cac cca cta tct tca ta 3' (SEQ ID NO.: 9) and Fiber2- 5' ggt gtc caa agg ttc gga ga 3' (SEQ ID NO.: 10). PCR reactions were performed as 95 °C 30 seconds; 54 °C 30 seconds; 72 °C 30 seconds for 30 cycles. All amplified products were analyzed on a 2% agarose gel.

For quantitative PCR, a molecular beacon based universal amplification and detection system was used (Intergen). A common leading sequence (Z sequence, 5' act gaa cct gac cgt aca 3') was added to the TPL1 and Fiber1 primers. The TPL2 and Fiber2 primers, described above, were used in the quantitative PCR reactions. 1 μl of the cDNA and one μg of Hirt DNA from each sample were used in the assay. The PCR were carried out in a 96-well spectrofluorometric thermal cycler (Applied Biosystems Prism 7700). The number of template molecules in the PCR reaction was calculated from the standard curve using linearized plasmid as templates.

As most late adenoviral genes transcripts share a common ~200 bp tripartite leader sequence (TPL) (Akusjarvi and Persson, Nature 292:420-6, 1981), the TPL sequence was chosen as a marker of viral gene expression. HepG2 cells were infected with the first generation vectors Ad2CMVGFP and Ad2HCRGFP, or the self-resolving vector, Ad2HCRGFP/EBV, using increasing multiplicities of infection. Total cellular RNA and low molecular weight DNA were isolated in parallel as described by Hirt (J. Mol. Biol. 26:365-9, 1967) and total RNA was prepared using RNAzol solution (Tel-Test. Inc.). RT-PCR was performed to quantitate the amount of RNA encoding the TPL in the cDNA samples. PCR amplification of a 201 bp fiber gene fragment from the AdV genome was used to detect the amount of viral genome in the DNA samples. A representative result of three experiments is shown in Figure 8A. TPL sequences were detected, 72 hours post-infection, with either 100 or 1000 viruses infected per cell, using both of the first generation adenoviruses (upper panel).

In contrast, no TPL signal was detected in the self-resolving Ad2HCRGFP/EBV infected cells, even at a moi of 100,000/cell. PCR amplification of the AdV fiber gene revealed comparable levels of AdV genomic DNA in cells infected at comparable moi's. (Figure 8A, lower panel). The cDNA samples in which the TPL signals were detected were further analyzed by real-time fluorescence PCR. The corresponding genomic DNA samples were also analyzed to determine the number of AdV genomes present in each sample. The results are summarized in Figure 8B. There were approximately $1x10^4$ TPL per $1x10^6$ AdV genomes detected in the Ad2HCRGFP infected cells, but no detectable TPL in the self-resolving Ad2HCRGFP/EBV infected cells. These results indicate that adenoviral gene expression was dramatically reduced by the separation of the viral enhancer sequences occasioned by the re-arrangement of the self-resolving vector.

TYPE A AND TYPE B VECTORS – EXPERIMENTAL RESULTS

Additional experimental examples now follow that further illustrate the general approaches of the invention relating to using and constructing type A and type B vectors. For generating such adenoviral vectors, DNA sequences important for gene expression in the target tissue were placed between two loxP sites. The first loxP site was inserted between the Ad2 left-end inverted terminal repeat (ITR) and the enhancer sequence, replacing a BspLU11I and BstZ17 fragment of Ad2. A target gene expression cassette, comprising a promoter, a gene of interest, polyadenylation signals, the EBV replicon, and site specific recombinase expression unit were inserted in place of the E1 locus.

In type A adenoviral vectors, the second loxP site is placed between TetR and VP16, preserving the coding frame of both (Figure 1A). A bidirectional promoter in which a central heptamer of tetracycline operator sites (TetO) (Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89:5547-5551, 1992) was flanked by two divergently oriented basal elements, directs the expression of TetR loxP VP16 from a synthetic TATA element, whereas Cre recombinase is controlled by the same heptamer of operator upstream of the HIV LTR basal element.

In the case of type B viruses (Figure 1B), the second loxP site was inserted in the first intron of the Ef1α gene, which contains the transcription stimulating sequences described herein. In addition, a splice acceptor sequence was added to the 5' end of the coding sequence of the gene of interest. To avoid rearrangement during plasmid

construction in bacteria, the Cre recombinase coding sequence was interrupted by the addition of the human IgG1 hinge-CH2 intron (between amino acids Q78 and A79), as described herein.

Designing a Compact EBV Replicon

Most plasmids employing the EBV latent origin of replication exceed 10 kb in length. To provide a means for increasing the capacity of the recombinant adenoviral type A or type B vectors to accommodate a therapeutic gene, a compact EBV replicon having episomal stability was designed. To this end, deletions were generated in both the cis-acting origin of replication, OriP, and the sequences encoding the trans-acting replication protein, Epstein Barr virus nuclear antigen-1 (EBNA-1) (Figure 9A). Episomal persistence was assessed with a green fluorescent protein (GFP)-bearing test plasmid by determining the fraction of cells retaining green fluorescence as a function of time, assuming that the half-life of GFP, in daughter cells that have not received an episome as a result of segregation failure, is approximately 1.4 days (Fukumura et al., Cell 94:715-725, 1998).

EBNA-1 contains a central repeated structure that consists entirely of Gly and Ala residues, termed the GA repeats (Figure 9A). Although deletion of this structure has been reported to have little consequence, a deletion mutant consisting of both a short OriP and a short EBNA-1 (SoriP + SEBNA1) was generated and found not to support plasmid maintenance effectively (approximately 40% loss per cell division). A version of this mutant, reconstructed with 40 GA repeats, in which the short OriP was paired with a short EBNA-1 provided significantly better plasmid stability (20% loss per cell division vs. 10 % per cell division for the wild type) (Figure 9B). Since most target tissues are relatively quiescent mitotically, this level of segregation fidelity provides reasonable stability in a compact replicon.

Producing Cell Lines that Express Cre- or FLP-Dominant Negative Mutants

As discussed herein, one obstacle to creating adenovirus carrying both recombinase and target sites has been the difficulty of controlling recombinase activity during virus propagation. Since efficient recombinase activity is needed in target cells, recombinase activity is best tempered in the production cell line.

Vector-independent methods to suppress recombinase activity during the production phase are attractive because they allow vector design objectives to be pursued with fewer constraints. In principle, dominant negative recombinase mutants provide the desired antagonism of recombinase activity. Cell lines expressing such recombinase dominant negative mutants were produced as follows.

Dominant negative Cre mutants were selected from known point mutants (Wierzbicki et al., J. Mol. Biol. 195:785-794, 1987) that are defective in recombination function but are likely to retain dimerization function Figures 10A-E. Several mutants were screened for their abilities to inhibit Cre activity of a type B vector construct (ad2239 in Figure 10B) in a transient cotransfection assay. Under these conditions, Cre activity is detected by the expression of GFP that occurs upon rearrangement. Figures 10D and 10E show the point mutants that were assessed and their relative activities in the transient cotransfection assay. Dilution studies, in which increasing amounts of substrate/Cre plasmid were cotransfected with the mutant forms, were conducted and based on its favorable profile, one mutant recombinase, designated CreY324C, was chosen for further development (Figure 10D).

Strong constitutive expression of CreY324C, under control of the Ef1α promoter failed to yield stable cell lines. Stable clones were obtained when the Ef1α promoter was replaced with a tetracycline regulated promoter (Gossen et al., Science 268:1766-1769, 1995). Clones were then tested for the ability to inhibit Cre enzymatic activities, and one clone, designated cell line #17, was selected for additional experiments. When a plasmid bearing Cre and capable of undergoing Cre-directed rearrangement to create a GFP transcription unit (ad2239) was transfected to #17 cells or parental 293ON cells, GFP expression in the #17 cells in the presence of 2μM doxycycline was significantly lower than those of controls (Figure 11), showing that Cre enzyme activity can be inhibited in #17 cells.

In addition to dominant negative Cre mutants, dominant negative FLP mutants may also be identified. FLP belongs to the same family of site-specific recombinases as Cre recombinase. A number of FLP mutations that show defects in either cleavage or ligation of FRT sites have been identified. Mutant FLP defective in cleaving FRT site (for example, H309L, L315P, G328R, G28E, N329D, S336Y, S336F, A339D, Y343F, and H345L) are generated using standard methods. Mutants that inhibit the wild type enzyme are then identified for generating stable cell lines according to the methods

described above. These and the other cell lines (described herein) are then used for producing FRT/FLP containing virus.

As mentioned above, difficulties creating stable cell lines expressing Cre dominant negative mutants were occasionally encountered. This difficulty is not limited to Cre mutants, but also to the wild-type Cre enzyme. In contrast, 293 cell lines stably expressing a thermostable FLP, referred to as FLPe (Buckholz et al., Nat. Biotechnol. 16:657-662, 1998), were created, suggesting that FLPe might not be as cytostatic as Cre protein. To demonstrate this, 293 cells were transfected with plasmids expressing either Cre or FLPe, and puromycin resistant colonies were selected. To generate stable cell lines expressing Cre or FLP mutants, 293 TetON cells were transfected with linearized plasmid expressing Cre or FLP mutants and puromycin acetyltransferase and selected with 1 μg/ml of puromycin. Puromycin resistant colonies were characterized further for their ability to inhibit Cre recombinase using the cre (ad2239) or flp (ad2879) substrate plasmids. Table 1 shows that there are more puromycin resistant colonies selected from FLPe transfected cells than from Cre transfected cells. From this result, it is expected that stable cell lines expressing a reasonably high level of dominant negative FLP may be readily created.

TABLE 1

Puromycin Resistant Colonies Formed When Cre Expressing or FLPe Expressing Plasmid was Used to Transfect 293 Cells

Expression Plasmid	Number of colonies (2 μg/ml puromycin)
Control	236
Cre	92
FLPe	127

Cre or Cre dominant negative mutants were also found to inhibit FLP activity (Table 2). Accordingly, cell lines such as cell line #17, that stably express a Cre dominant negative mutant (for example, CreY324C), are useful for producing FLP/FRT carrying adenovirus.

TABLE 2

Cre Inhibition of FLP Activity *in trans*

Plasmids	Arbitrary GFP intensity
Ef1α FLP + FLP substrate + vector control	4.9
Ef1α FLP + FLP substrate + Ef1α Cre	0.78
Ef1α FLP + FLP substrate + Ef1α Cre R173C	2.23

FLP enzyme activity was measured by the GFP intensity by cotransfecting with a FLP substrate plasmid, ad2879 (Figure 19A). GFP intensity was quantified using IP lab software.

Transcriptional Regulation of Cre or FLP Recombinases

It has been relatively difficult to achieve high-level promoter inducibility in a replicating adenovirus. The challenge is similar to that of achieving faithful control of transcription in a transient expression setting. One approach to increase the induction ratio in a transient setting is the use of auto-regulatory (feed-forward) circuits. One such system, based on tetracycline dependent activation, is shown in Figure 12. A central heptamer of tetracycline promoter operator sites (TetO sites) was placed between two divergently oriented basal TATA elements. The leftward TATA controls the expression of the TetR-VP16 fusion protein, in which a loxP (or FRT) site has been placed between the TetR DNA binding domain and the VP16 transcriptional activator. The rightward TATA box directs the synthesis of recombinase, either Cre or the yeast FLP enzyme. In the presence of tetracycline, the promoter has reduced activity in both directions. Upon removal of tetracycline, the synthesis of both TetR-VP16 and recombinase are induced (Figure 1A). The induced recombinase then disjoins the TetR DNA binding element from the transcriptional activation contributed by VP16. Any existing TetR-VP16 fusions thereafter promote transcription of TetR, which competes with TetR-VP16 for TetO, resulting in deinduction of recombinase transcription.

When a model target cell line, HepG2, was tested with this type of adenovirus, the efficiency of circularization was low relative to that seen in 293 cells (data not shown), indicating a cell dependence of the bidirectional TetO promoter. To correct this, the TATA element of the TetO synthetic promoter (derived from the CMV immediate early promoter) was replaced with that of the HIV LTR. Constructs bearing differing components of the HIV basal promoter were analyzed for strength and regulation in 293 and HepG2 cells (Figure 13A). Among the constructs tested, one

version bearing the HIV LTR TATA and Sp1 elements (D in Figure 13A) showed the least basal expression in 293 cells (data not shown) and the greatest induction in HepG2 cells (Figure 13B).

Using this promoter, a construct (ad3400) containing the autoregulatory structure D as shown in Figure 13A was engineered, and Cre activities in the presence and in the absence of tetracycline were assayed. Plasmid ad2265 (Figure 14) in which a blue fluorescent protein (BFP) expression unit is interrupted by two loxP sites and transcription termination sequences was used as a substrate for Cre. Cre-mediated recombination joins BFP to the promoter resulting in BFP expression. As shown in Table 3, no difference was found in the intensity of BFP expression, either in the presence or absence of tetracycline. One possible explanation for this is that very little Cre protein is required for activity. Consistent with this idea, standard imunohistochemical techniques failed to reveal the presence of Cre enzyme in cells that were fully induced (data not shown).

TABLE 3

Cre Recombinase Activity Regulation in Type A Constructs

Construct	Cre Form	+tet -tam	-tet -tam	+tet +tam	-tet +tam
ad3400	Cre	2.58	3.39	2.71	6.20
ad4394	Cre-LBD	0.081	0.22	0.056	1.02
ad4705	LBD-Cre-LBD	ND	ND	ND	ND

Cre enzyme activity was measured in the presence or absence of the ligands, tamoxifen, by cotransfecting with the substrate plasmid (ad2265). BFP intensity (mean intensity/area) was quantified by analyzing fluorescent images captured by a digital camera using IP lab software. ND, refers to fluorescent intensities that were too weak to measure. Tet, Tetracycline; tam, tamoxifen.

Deletion of the PolyA Consensus Sequence from Cre or FLP Transcription Units

To reduce the expression of FLP or Cre recombinase further, the consensus polyA addition signals from the Cre or FLP transcript unit were deleted from vector constructs, leaving polyadenylation dependent on distal downstream sequences, for example, in gene IX. The activity of Cre using type B proviral constructs with or without the polyA signal was measured. As shown Table 4, the construct without polyA signals (AD229.3) showed a significant reduction of GFP intensity compared to a

construct bearing the polyA signal (AD230.5). When FLPe constructs of similar structure were evaluated, similar results were found (data not shown). These data show that Cre and FLPe enzyme activity levels can be modulated by attenuating polyadenylation.

TABLE 4

Effect of Deleting polyA Addition Signal From the Cre Expression Unit on Cre Enzyme Activity Level

	polyA	Relative GFP Intensity
AD229.3	-	0.25
AD230.5	+	1

Post-Transcriptional Regulation of Cre Recombinase Activity

Post-transcriptional control mechanisms of Cre recombinase activity were also evaluated. Translational fusions between Cre and the ligand binding domain (LBD) of estrogen receptor have been reported to be regulated by estrogen (Feil et al., Proc. Natl. Acad. Sci., U.S.A 93:10887-10890, 1996; Gossen et al., Proc. Natl. Acad. Sci., U.S.A. 89:5547-5551, 1994), or, in the case of mutant estrogen receptors (Metzger et al., Proc. Natl. Acad. Sci. U.S.A. 92:6991-6995, 1995), by the partial antagonist tamoxifen.

Use of a ligand-dependent recombinase (ad4394 in Table 3), in combination with the HIV LTR-based autoregulated Tet system, allowed for a small degree of regulation by tetracycline, but not by ligand, as assayed using the ad2265 rearrangement assay (Table 3). One interpretation of this finding is that fusion of the estrogen receptor LBD to Cre provides only modest control of recombinase activity, but attenuates enzyme potency to a level so that transcriptional regulation can be measured.

To increase control of recombinase activity, the LBD was fused both to the N-terminus and C-terminus of Cre (LBD-Cre-LBD) and inserted into the coding sequence of both type A and type B vectors. When the LBD-Cre-LBD construct of type A was transfected into 293 cells, it showed no significant Cre enzyme activity even in the presence of ligand (Table 3). This result confirmed that the Cre recombinase activity is attenuated by N-terminal or C-terminal extension.

When the LBD fusion Cre enzymes were assayed in the type B vector context, only LBD-Cre-LBD fusions (pk8-ad4626) showed ligand-dependent regulation of Cre enzyme activities (Table 5). It appears that attenuated Cre activity in LBD-Cre-LBD, in the absence of ligand, is low enough to fall below the upper limit of the Cre assay.

TABLE 5

Cre Enzyme Activities of Type B Provirus

Cre Form	Provirus	-tam	+tam
Cre	pk8-ad2239	ND	ND
Cre-LBD	pk8-ad4332	4.1	6
LBD-Cre-LBD	pk8-ad4626	0.05	4.2

Cre enzyme activity was measured in the presence or absence of the ligands, tamoxifen. GFP intensity was quantified using IP lab software.
tam, tamoxifen.

Consistent with this notion, only the construct carrying two LBDs, pk8- ad4626, was able to produce virus (AD121.5) by transfection and propagate in 293 cells, while pk8-ad4332, which carried one LBD, produced virus (AD100.9) initially (following transfection of the cognate DNA) but was unable to propagate in 293 cells (Table 6). In the case of wild type Cre, no virus was produced in 293 cells by transfection.

TABLE 6

Production and Propagation of Type B Adenovirus

Cre Form	Type B adenovirus	Viral Production in 293 cells	Viral Propagation in 293 cells	Viral Propagation in #17 cells
Cre	Pack8-2239	-	-	-
Cre-LBD	AD100.9 (Pack8-4332)	+	-	+
LBD-Cre-LBD	AD121.5 (Pack8-4626)	+	+	+

The AD100.9 virus was able to propagate in #17 cells expressing the dominant negative Cre Y324C, demonstrating that modulation of Cre activity is important for viral production. Thus, adenovirus carrying both two loxP sites and Cre in two different configurations were generated by controlling Cre activity.

Viral Rearrangement in Culture

Cre/loxP mediated rearrangement of the adenovirus in tissue culture cells has also been analyzed. As shown in Figure 15A, the AD121.5 virus showed a significant increase in GFP expression in the presence of the ligand, estrogen, suggesting a successful rearrangement of the virus by Cre recombinases. When non-chromosomal DNA (Hirt, *J. Mol. Biol.* 40:141-144, 1969) was made from the cells and analyzed by DNA blot analysis, the viral DNA from estrogen treated cells was identified mostly in circular form (C in Figure 15B), while the DNA from cells not treated with estrogen was found mainly in linear form (L in Figure 15B).

To evaluate the efficiency of the self-rearranging viruses *in vivo*, high titer stocks of AD102.7 (a type A virus carrying LBD-Cre, pk8-ad4394) in #17 cells was prepared and purified by CsCl gradient ultracentrifugation. The titer of AD102.7 (4 –6 x 10^{12}/ml by OD) is comparable to or slightly exceeds that of control viruses (2 –4 x 10^{12}/ml by OD) which carry neither Cre nor a loxP site. To determine the efficiency of viral rearrangement *in vivo* and whether such rearrangement is dependent on the presence of ligand, AD102.7 virus (4 x 10^{11} pfu/mouse as determined by optical density) were injected via tail vein into Rag-2 mice that were pretreated with vehicle alone or 110 μg/day of tamoxifen for 7 days as follows.

Rag-2 mice were injected with either PBS (mock) or 4 X 10^{11} adenovirus particles (as determined by OD_{260}) of type A virus, AD102.7, via the tail vein. At various times after injection, animals were sacrificed and the liver tissues were removed and frozen rapidly on dry ice. To visualize GFP expression in animal tissues, mice were anaesthetized and perfused with 4% paraformaldehyde containing 0.2% glutaraldehyde intracardially (Kafri et al., Natl. Genet. 17:314-317, 1997), and the liver tissues were removed and fixed overnight at room temperature in the perfusion buffer containing 30% sucrose. The fixed tissues were sectioned serially and observed under confocal scanning laser microscopy. In experiments evaluating the responses of ligand-regulated recombinase, mice were injected either with vehicle (vegetable oil) alone or with 110 μg/day of tamoxifen for 7 days prior to adenoviral injection.

Liver tissues from these animals were harvested at 2.5 hrs post injection (the earliest time point taken after injection) and Hirt DNA from approximately 250 mg of frozen hepatic tissue was prepared and analyzed by blot analysis. As shown in Figure 16, the majority of adenoviral DNA was found in circular form in tissues from untreated mice, as well as tamoxifen-treated mice. It can be concluded from these data that the Cre enzyme activity present in the tissue, even in the absence of ligand was sufficient for efficient self rearrangement of virus. As expected, the hepatic tissues from the Rag2 mice injected with AD102.7 showed strong expression of GFP (Figure 17).

Demonstrating that AD102.7 virus, produced efficiently in 293 cells at high titres by the conventional means, can self rearrange efficiently *in vivo* provides the proof of the concept that potentially safer adenoviral gene therapy vectors can be produced.

Adenoviruses Carrying Both FRT and FLP Recombinase

Type A and type B proviral constructs carrying both FRT (FLP recombinase recognition site) and FLP recombinase were also generated. Structures of these viruses are analogous to those of loxP/Cre carrying viruses except that loxP sites are replaced by FRT sites and Cre coding sequence is replaced by FLP coding sequence (Figures 18A, 18B).

Virus Production at Reduced Temperature

Temperature dependence of the Ef1α promoter using GFP expression as a marker was also examined. As shown in Table 7, Ef1α promoter activity is strongly reduced at 32°C in comparison to 37°C or 39°C. The temperature sensitive nature of the Ef1α promoter was used to propagate type B adenovirus carrying FLP at 32°C following initial production of the virus by DNA transfection (pk8-ad3302) at 37°C. HepG2 cells infected with these viruses (AD41.4) showed strong GFP expression, but with an approximately 12 hr delay compared to GFP expressing viruses, suggesting that FLP recombinase activity may be impaired at 37°C. To improve the activity of FLP recombinase, viral constructs were created using a thermostable FLP (referred to as "FLPe") described by Buchholz *et al.* (Nat. Biotechnol. 16:657-662, 1998).

TABLE 7

Effects of Temperature on Ef1α Promoter Strength as Shown by GFP Intensities

Tester plasmids		Arbitrary GFP Intensities		
		32°C	37°C	39°C
Ef1α GFP	16 hrs	1475	7886	11409
	41 hrs	6472	36699	50787
	86 hrs	16256	53370	54424
Ef1α Cre +ad2204	16 hrs	243	1141	2132
	41 hrs	1094	9119	9784
	86 hrs	695	3219	8144

GFP intensities were measured using a Fluorescent reader.

The activities of FLP and FLPe using a FLP substrate plasmid (ad2879, Figure 19A) in 293 cells were compared. As shown in Table 8, FLPe is significantly more active than FLP under these conditions.

TABLE 8

FLPe is Significantly More Active than FLP Recombinase

Plasmid		Mean GFP intensity
ad4821	Ef1α FLPe	2.39
ad2949	Ef1α FLP	0.01

Plasmid coding either FLPe or FLP was cotransfected with a FLP substrate plasmid (Figure 19A) into 293 cells. GFP intensity of each transfection was measured using IP lab program.

In addition, a tamoxifen-regulated FLPe was created by fusing the ligand-binding domain from a mutant form of estrogen receptor to the FLPe coding sequence at its C-terminus (FLPe-LBD). The FLPe-LBD was found to be regulated by the ligand, tamoxifen (Table 9). Although FLP activity was retained by C-terminal fusion (FLP-LBD), addition of a short oligopeptide tag to the N-terminus of FLP abolished its activity (data not shown).

TABLE 9

Tamoxifen Regulation of FLPe as Determined by GFP Intensities

Plasmid	FLPe	-tam	+ tam
ad4821 +ad2879	FLPe	++++	++++
ad5022 +ad2879	FLPe-LBD(tam)	+	+++

GFP intensity resulting from FLPe mediated recombination was measured using a fluorescent microscope. tam, 2 μg/ml tamoxifen.

Inhibition of FLPe Activities by Anti-sense FLPe

An anti-sense approach to inhibit FLP enzyme activity was also employed. This approach tested the notion that incorporation of an open reading frame into an antisense transcript would stabilize the transcript and potentiate antisense activity. Two approaches were utilized. In one approach, the BFP coding sequence was placed upstream of anti-FLPe. In the second approach, an anti-FLPe was placed upstream of an internal ribosome entry sequence (IRES) and the BFP coding sequence (Figure 20). The ability of these constructs to inhibit FLPe was assayed using a FLP substrate plasmid, ad2879 (Figure 19A) and the result is shown in Figure 21. These data show that anti-sense FLPe is more effective in inhibiting FLPe function when it is fused to BFP, which can presumably be replaced with any other stable protein.

OTHER SELF-REARRANGING ADENOVIRUSES

Mixed Infection With Adenoviruses Carrying loxP/FLP and FRT/Cre

One of the ways to produce adenoviruses that can be rearranged in target cells but not producer cells is to engineer two separate viruses, each carrying one recombinase and the target sequence for the other. To test this system, type B adenoviral constructs carrying Cre recombinase and FRT sites and FLPe recombinase and loxP sites were created (Figure 22). In target cells infected with both viruses, Cre catalyzes recombination between the two loxP sites in the FLP virus, and FLP carries out FRT mediated recombination in the Cre virus, resulting in two circular plasmids. The loxP virus contained BFP, whereas the FRT virus contained GFP. Measurement of the fluorescent intensities of GFP and BFP, after cotransfecting the two constructs, revealed that BFP expression (mediated by Cre enzyme) was greater than GFP expression

(mediated by FLP enzyme), suggesting that the Cre enzyme functions more efficiently than FLP.

Accordingly, these two recombinase activities in the target cells need to be balanced for complete circularization of both viral vectors. Exemplary methods for modulating Cre /FLP activity include the use of transcriptional regulation (such as by varying promoter strength and/or with or without poly A addition signal sequence) and translational and/or post-translational regulation (such as by changing FLP to FLPe and making LBD fusion proteins), and post viral production control (such as by changing the ratio of two viruses).

In one approach, Cre was replaced by Cre-LBD and FLP was replaced by FLPe. To improve identification of the rearrangement products, BFP was replaced with RFP as a marker for Cre recombination. As shown in Table 10, in the presence of estrogen, expression of GFP (FLPe mediated) and RFP (Cre mediated) were similar.

TABLE 10

RFP and GFP Expression of Cells Cotransfected With Type B Proviral Constructs Carrying Cre-LBD/FRT(GFP) or FLPe/loxP (RFP)

Plasmids	Genotypes	GFP intensity		RFP intensity	
		Estrogen			
pk8-ads120 +pk8-ads113	Cre-LBD/FRT (GFP) + FLPe/loxP (RFP)	-	+	-	+
		3222	3183	46	1954

To insure that both Cre and FLP carrying viruses with an optimal ratio infect each target cell, these viruses can be cross-linked prior to infection. For example, Cre carrying virus is labeled by biotin while FLP carrying virus is labeled by avidin. Mixing two types of modified viruses generates virus complexes of desired proportions as well. Biotinylation or avidinylation can be carried out using commercially available reagents such as EZ-Link TFP-PEO biotin (Pierce) and EZ-Link maleimide activated NeutrAvidin (Pierce). The extent of the biotin/virus and avidin/virus will be empirically determined to ensure the viability of the virus and to obtain an optimal ratio of two viruses in the complex. Optimal ratios will be those resulting in 1:1 Cre and FLP recombinase activities in target cells. The modifications will be done following manufacture's instructions.

This approach not only increases the effective capacity of adenoviral vector but also opens new avenue of applications involving multiple proteins, some of which cannot be coexpressed in production cell line as a result of combination toxicity.

All references mentioned herein are hereby incorporated by reference. Other embodiments are within the claims.

What is claimed is:

Claims

1. A replicatable viral DNA vector encoding a site-specific DNA-altering enzyme and a DNA target recognized by said enzyme, said enzyme selectively converting, in a cell expressing said enzyme, said DNA vector to a rearranged form.

2. The vector of claim 1, wherein said rearranged form comprises an autonomously replicating episome.

3. The vector of claim 1, wherein said rearranged form comprises linear and circular DNAs.

4. The vector of claim 1, wherein said vector comprises adenoviral DNA.

5. The vector of claim 1, wherein said vector comprises a genetically-engineered recombination site.

6. The vector of claim 5, wherein said recombination site comprises a target of Cre or FLP.

7. The vector of claim 1, wherein said enzyme comprises a recombinase or an integrase.

8. The vector of claim 7, wherein said recombinase is Cre or FLP recombinase.

9. The vector of claim 1, wherein said enzyme is functional in a mammalian cell.

10. The vector of claim 5, wherein said recombination site comprises a recognition sequence of a site-specific DNA-altering enzyme.

11. The vector of claim 1, wherein said vector comprises an origin of replication functioning in a mammalian cell.

12. The vector of claim 11, wherein said origin of replication is an Epstein Barr Virus replicon.

13. The vector of claim 1, wherein said vector comprises a gene of interest.

14. A method for assembling a recombinant adenoviral DNA said method comprising the steps of: (a) providing a first linearized DNA vector comprising a restriction site and a cos site and a second linearized DNA vector comprising said restriction site, an adenoviral nucleic acid molecule, and a cos site; and (b) ligating said first and second linearized DNA vectors, said ligation assembling a recombinant adenoviral DNA.

15. The method of 14, wherein said first linearized DNA vector comprises a selectable marker.

16. The method of claim 14, wherein said first linearized DNA vector comprises an adenoviral left end-inverted terminal repeat.

17. The method of claim 14, wherein said first linearized DNA vector comprises a gene of interest.

18. The method of claim 14, wherein said second linearized DNA vector comprises a selectable marker.

19. The method of claim 14, wherein said second linearized DNA vector comprises an adenoviral right-end inverted terminal repeat.

20. The method of claim 14, said method further comprising packaging said assembled adenoviral DNA into a phage and infecting a host cell.

21. The method of claim 14, wherein said first and second linearized DNAs comprise a cosmid vector.

22. The method of claim 14, wherein said adenoviral DNA is flanked by cleavage sites.

23. The method of claim 22, wherein said cleavage sites comprise intron endonuclease cleavage sites.

24. An adenovirus producer cell comprising a nucleic acid molecule that expresses a dominant negative site-specific DNA-altering enzyme.

25. The producer cell of claim 24, wherein said site-specific DNA altering enzyme is a dominant negative recombinase.

26. The producer cell of claim 25, wherein said recombinase is a Cre or Flp recombinase.

27. The producer cell of claim 26, wherein said dominant negative recombinase is CreY324C.

28. The producer cell of claim 26, wherein said Flp recombinase is Flpe.

29. The producer cell of claim 24, wherein said cell is a 293 human embryonic kidney cell.

30. A vector comprising, in the 5' to 3' direction,

a first genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme;

a gene of interest;

a lineage-specific gene promoter;

a second genetically engineered *cis*- acting target recognized by a site-specific DNA altering enzyme; and

a nucleic acid molecule encoding a site-specific DNA altering enzyme.

31. A vector comprising, in the 5' to 3' direction,

a first genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme;

a gene of interest;

a bi-directional promoter, comprising a second genetically engineered *cis*-acting target recognized by a site-specific DNA altering enzyme; and

a nucleic acid molecule encoding a site-specific DNA altering enzyme.

32. A method of gene therapy comprising the administration to a patient in need of gene therapy a therapeutically effective amount of the vector of any one of claims 1, 30, or 31 which is expressed in said patient

33. A population of cells transfected with the vector of any one of claims 1, 30, or 31.

34. A method of gene therapy comprising the administration to a patient in need of gene therapy a therapeutically effective amount of the population of cells of claim 33.

FIG. 1A

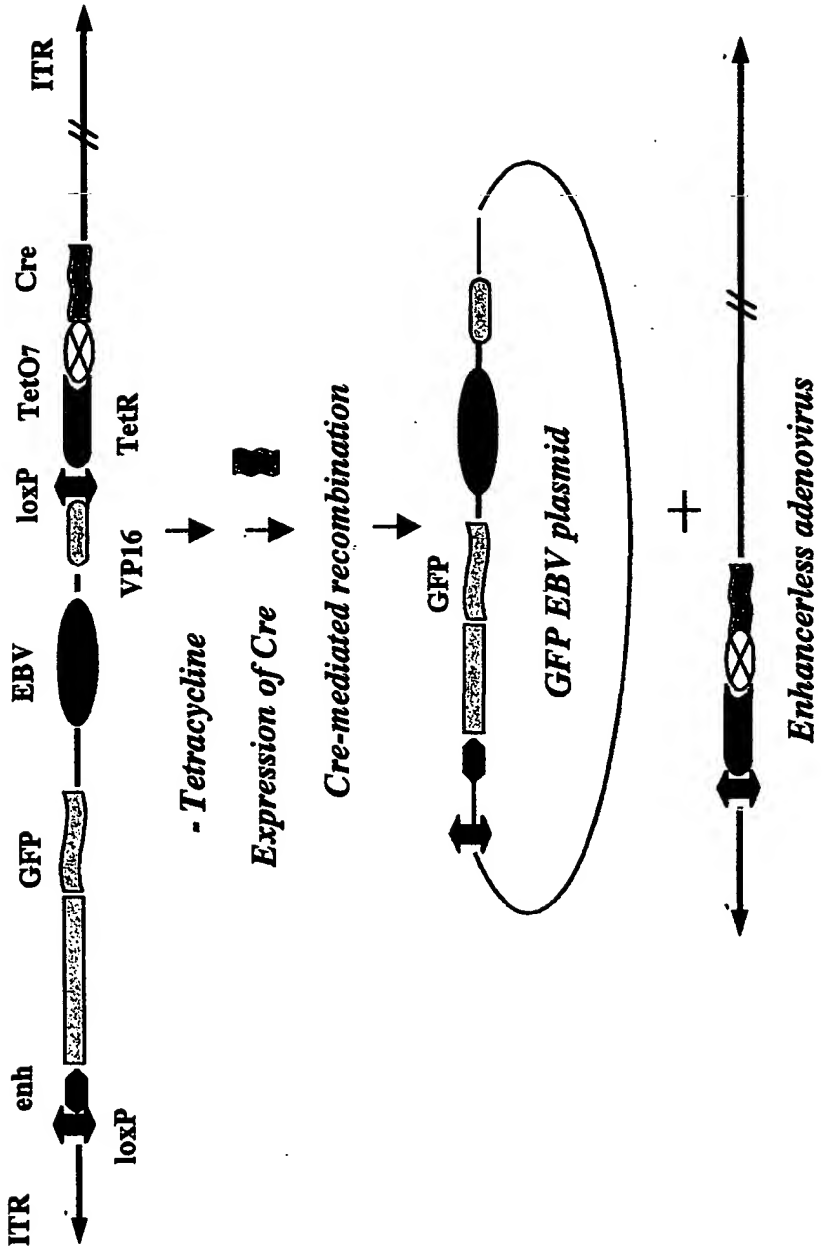

ITR
enh
loxP
GFP
EBV
loxP
TetO7
Cre
ITR
VP16
TetR
- Tetracycline
Expression of Cre
Cre-mediated recombination
GFP
GFP EBV plasmid
+
Enhancerless adenovirus

FIG. 1B

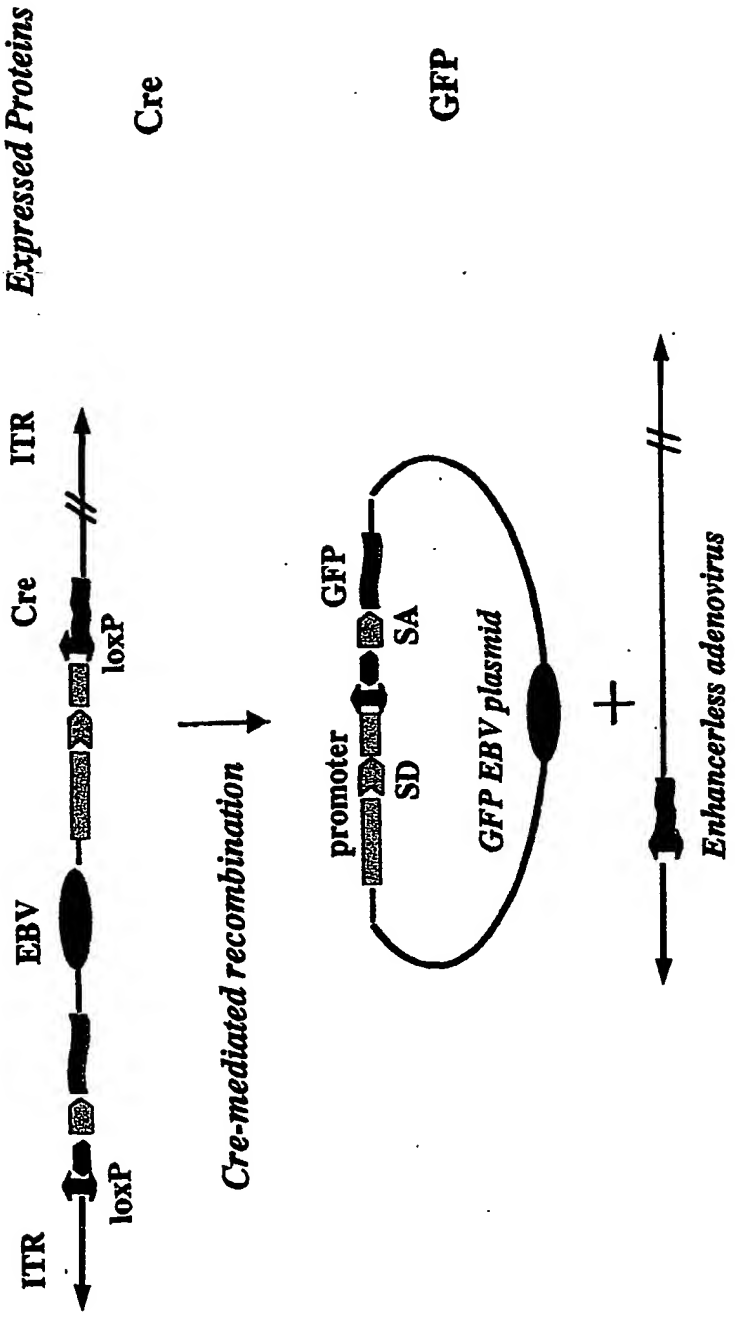

Expressed Proteins
Cre
GFP
ITR
Cre
EBV
loxP
loxP
ITR
Cre-mediated recombination
GFP
SA
promoter
SD
GFP EBV plasmid
Enhancerless adenovirus

FIG. 2A

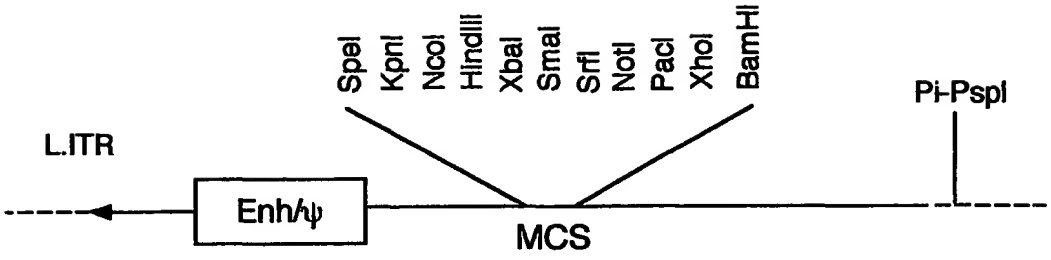
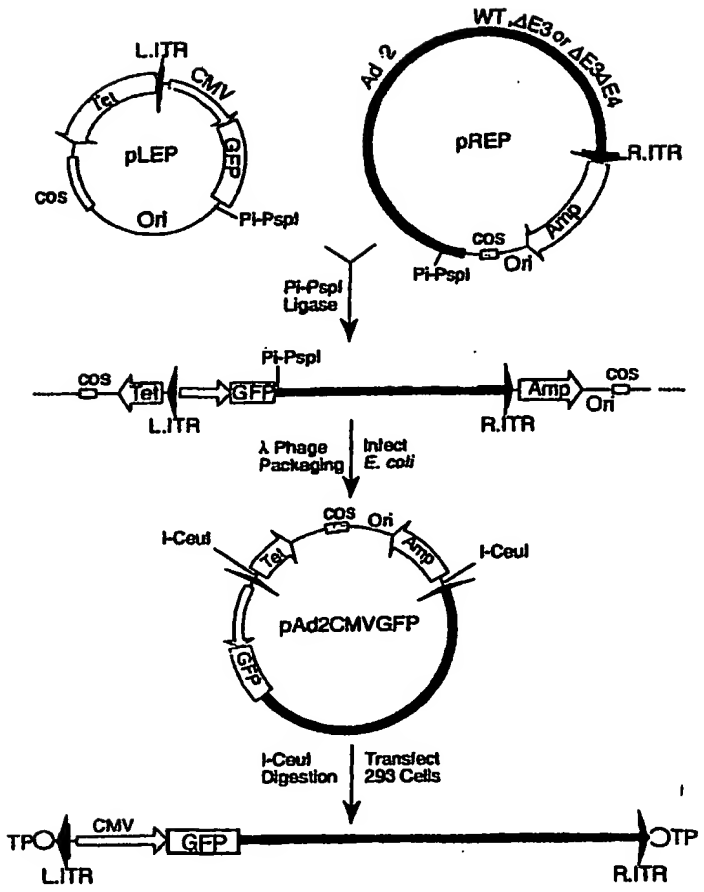

SpeI
KpnI
NcoI
HindIII
XbaI
SmaI
SrfI
NotI
PacI
XhoI
BamHI
Pi-PspI
L.ITR
Enh/ψ
MCS

FIG. 2B


L.ITR
CMV
Tet
GFP
pLEP
cos
Ori
Pi-Pspl
WT, ΔE3 or ΔE3/E4
Ad 2
pREP
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cos
Amp
Ori
Pi-Pspl
Pi-Pspl Ligase
Pi-Pspl
cos
Tet
GFP
L.ITR
Amp
cos
Ori
R.ITR
λ Phage Packaging
Infect E. coli
cos
Ori
I-CeuI
Tet
Amp
I-CeuI
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GFP
I-CeuI Digestion
Transfect 293 Cells
TP
CMV
GFP
TP
L.ITR
R.ITR

FIG. 3A

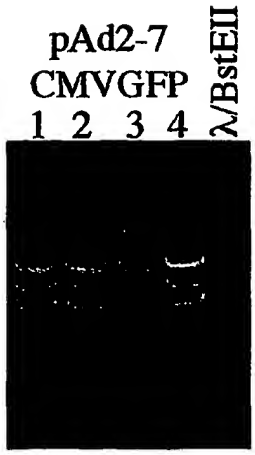


FIG. 3B

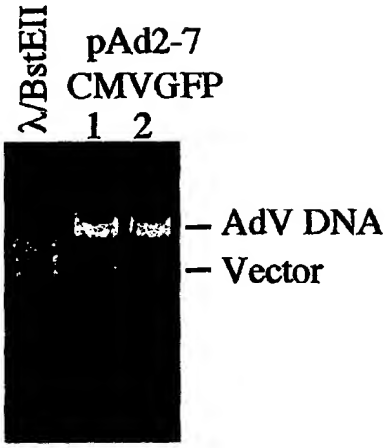
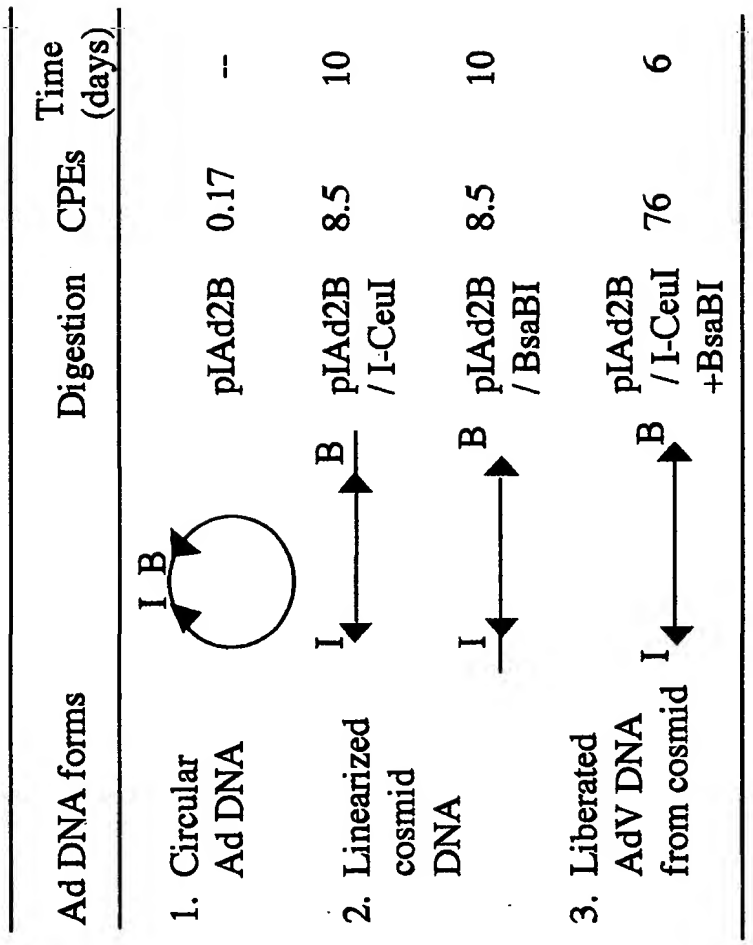


FIG. 4A


Ad DNA forms
Digestion
CPEs
Time (days)
1. Circular Ad DNA
I B
pIAd2B
0.17
--
2. Linearized cosmid DNA
I
B
pIAd2B / I-CeuI
8.5
10
I
B
pIAd2B / BsaBI
8.5
10
3. Liberated AdV DNA from cosmid
I
B
pIAd2B / I-CeuI +BsaBI
76
6

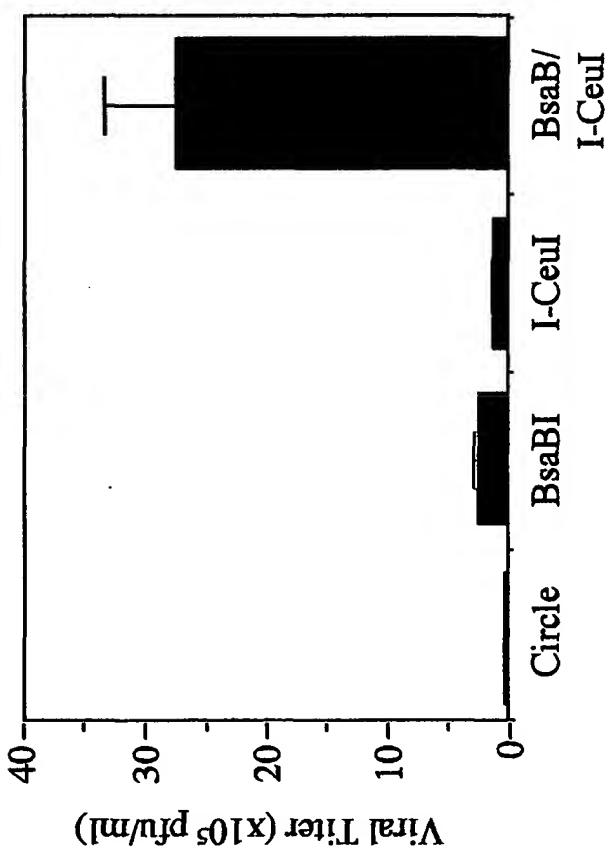
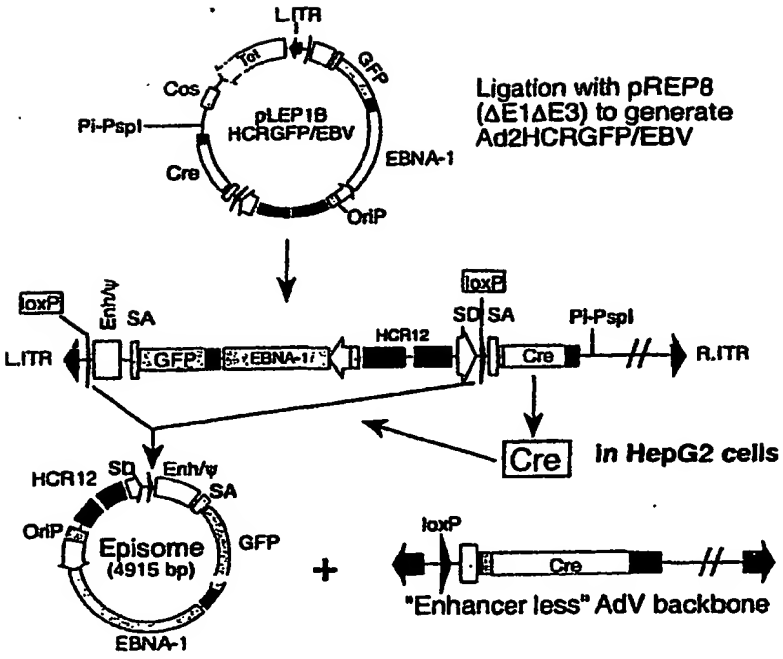

Viral Titer (x10^5 pfu/ml)
0
10
20
30
40
Circle
BsaBI
I-CeuI
BsaB/
I-CeuI

FIG. 4B

FIG. 5


L.ITR
Tat
GFP
Cos
pLEP1B
HCRGFP/EBV
PI-PspI
EBNA-1
Cre
OriP
Ligation with pREP8
(ΔE1ΔE3) to generate
Ad2HCRGFP/EBV
loxP
Enh/ψ
SA
SD
HCR12
PI-PspI
L.ITR
GFP
EBNA-1
Cre
R.ITR
Cre
in HepG2 cells
HCR12
SD
Enh/ψ
SA
OriP
Episome
(4915 bp)
GFP
EBNA-1
+
loxP
Cre
"Enhancer less" AdV backbone

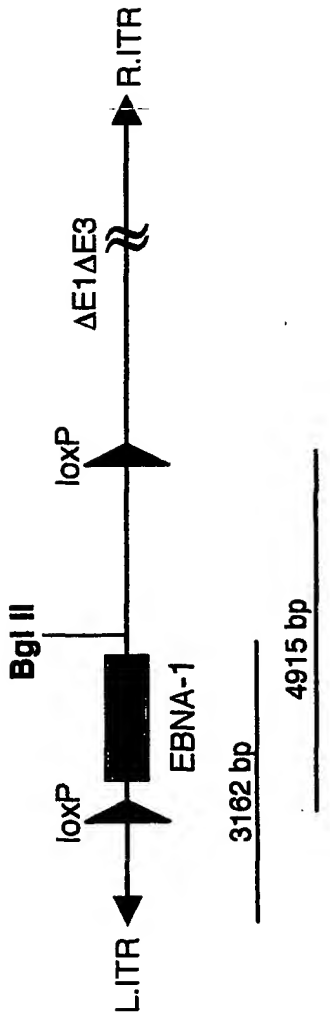


FIG. 6A

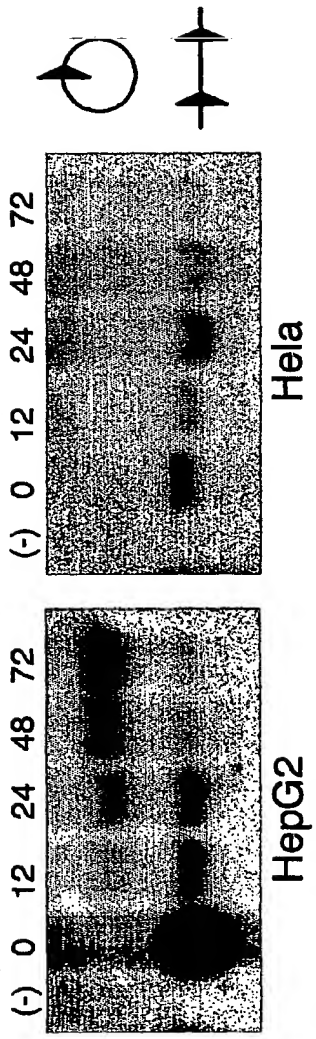


FIG. 6B

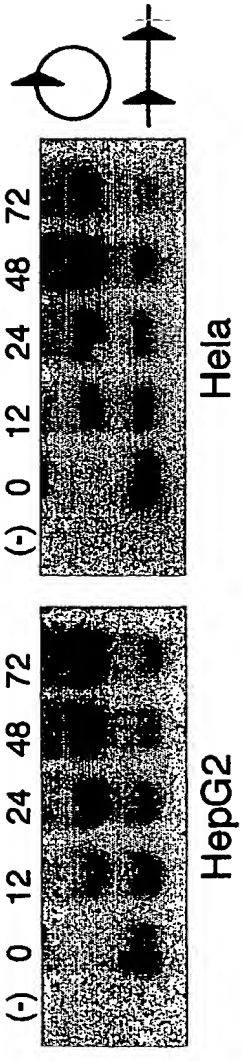


FIG. 6C

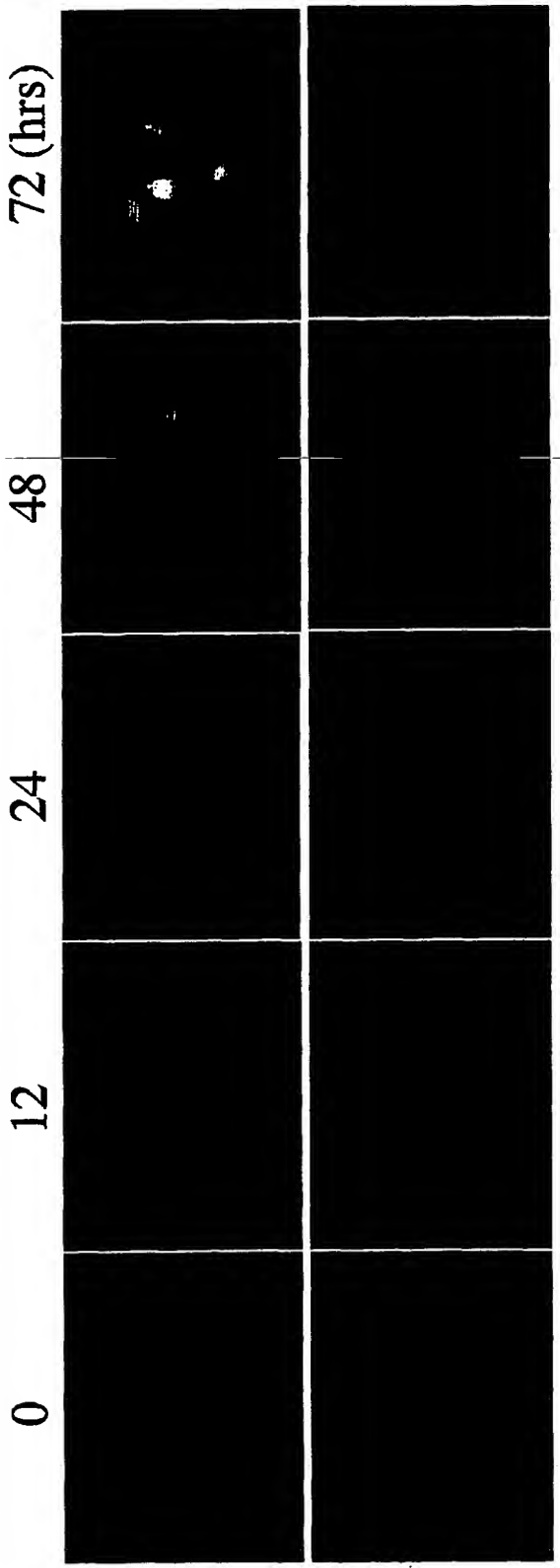


FIG. 7A

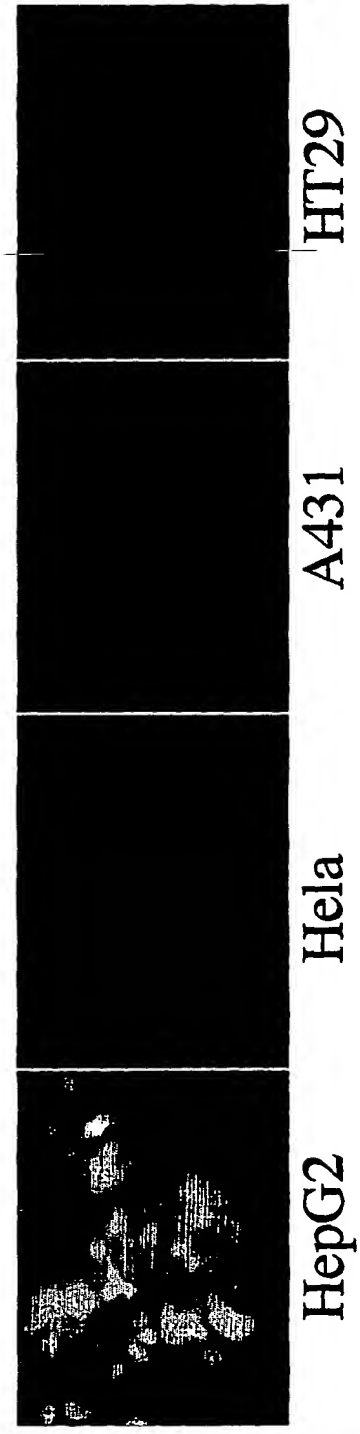
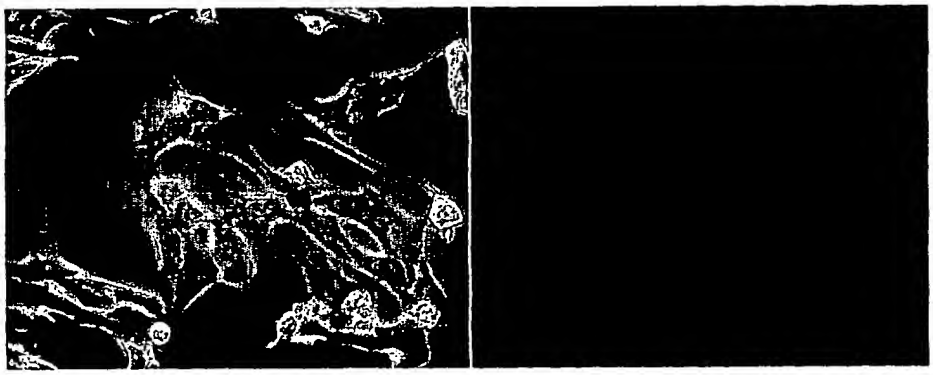


FIG. 7B

FIG. 7C



Human primary hepatocytes

FIG. 8A

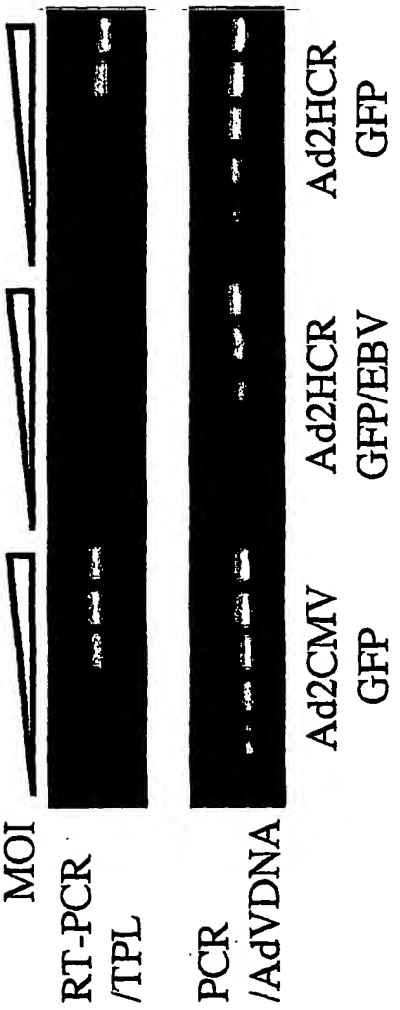

MOI
RT-PCR
/TPL
PCR
/AdVDNA
Ad2CMV
GFP
Ad2HCR
GFP/EBV
Ad2HCR
GFP

FIG. 8B

	# of TPL mRNA / 10^6 AdV DNA
Ad2HCRGFP	1.15 ± 0.28 x 10^4
Ad2HCRGFP/EBV	Non-detectable

FIG. 9A

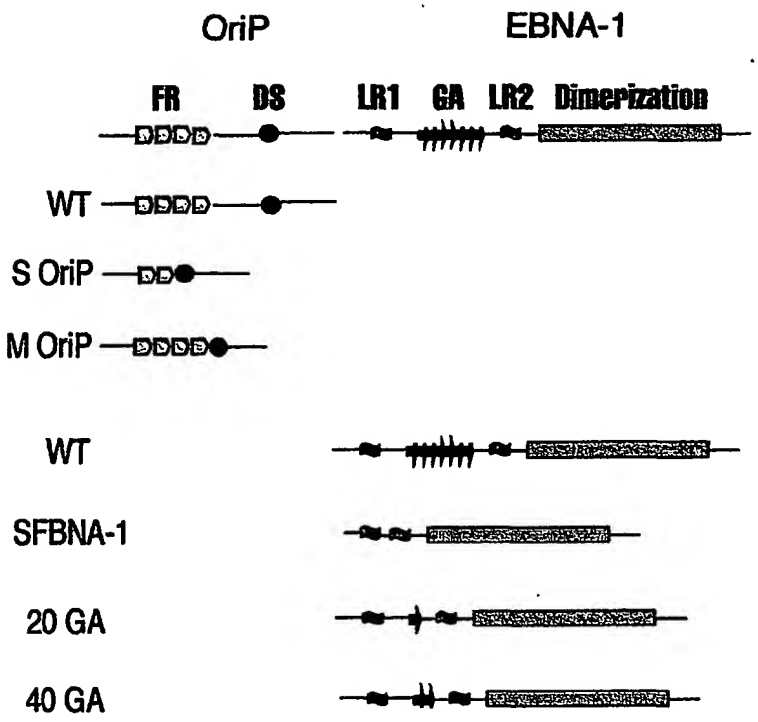

OriP
EBNA-1
FR
DS
LR1
GA
LR2
Dimerization
WT
S OriP
M OriP
WT
SFBNA-1
20 GA
40 GA

FIG. 9B

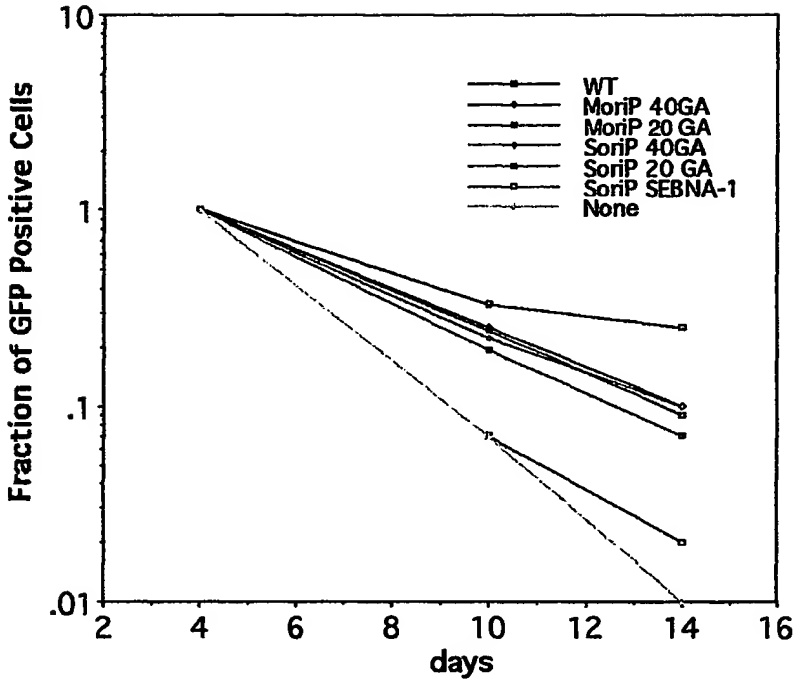

10
1
.1
.01
Fraction of GFP Positive Cells
2
4
6
8
10
12
14
16
days
WT
MoriP 40GA
MoriP 20 GA
SoriP 40GA
SoriP 20 GA
SoriP SEBNA-1
None

FIG. 10A

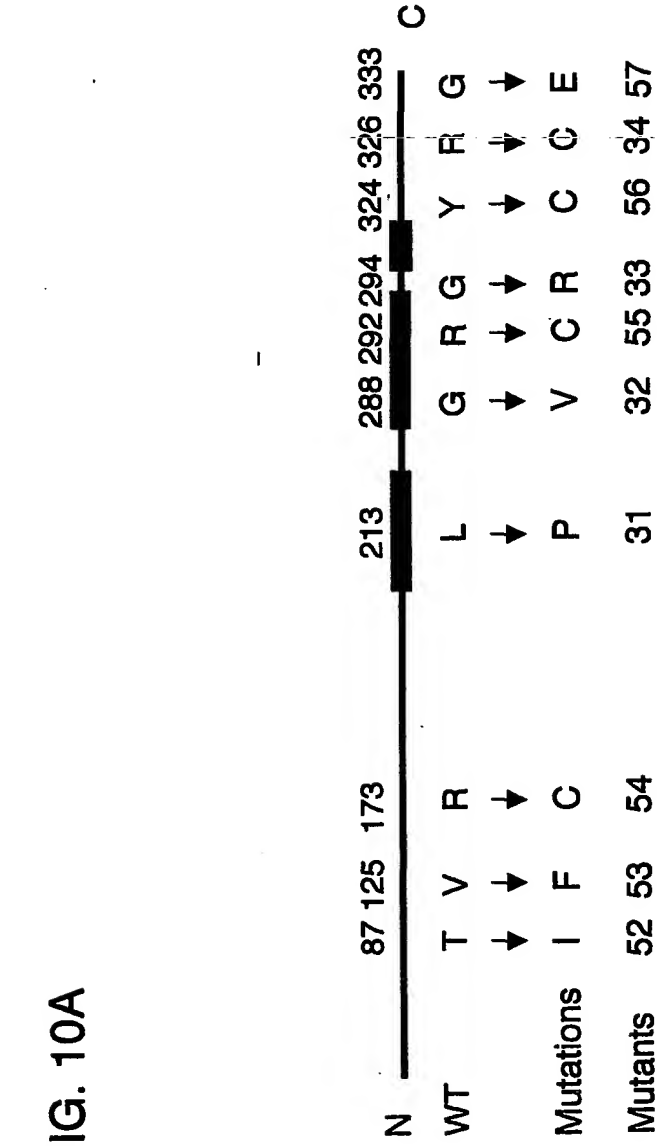

N
C
87 125 173
213
288 292 294
324 326 333
WT
T V R
L
G R G
Y R G
Mutations
I F C
P
V C R
C C E
Mutants
52 53 54
31
32 55 33
56 34 57

FIG. 10B

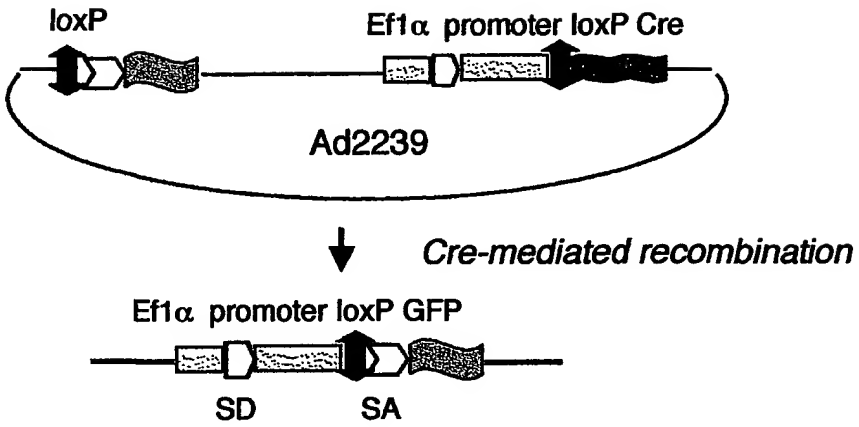

loxP
Ef1α promoter loxP Cre
Ad2239
Cre-mediated recombination
Ef1α promoter loxP GFP
SD
SA

FIG. 10C

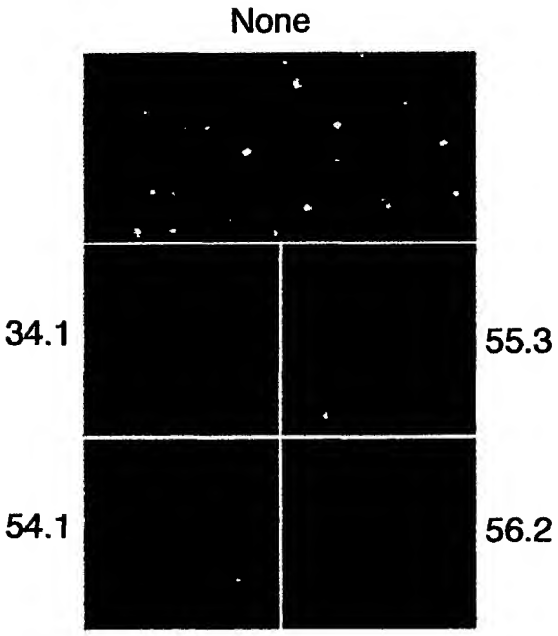
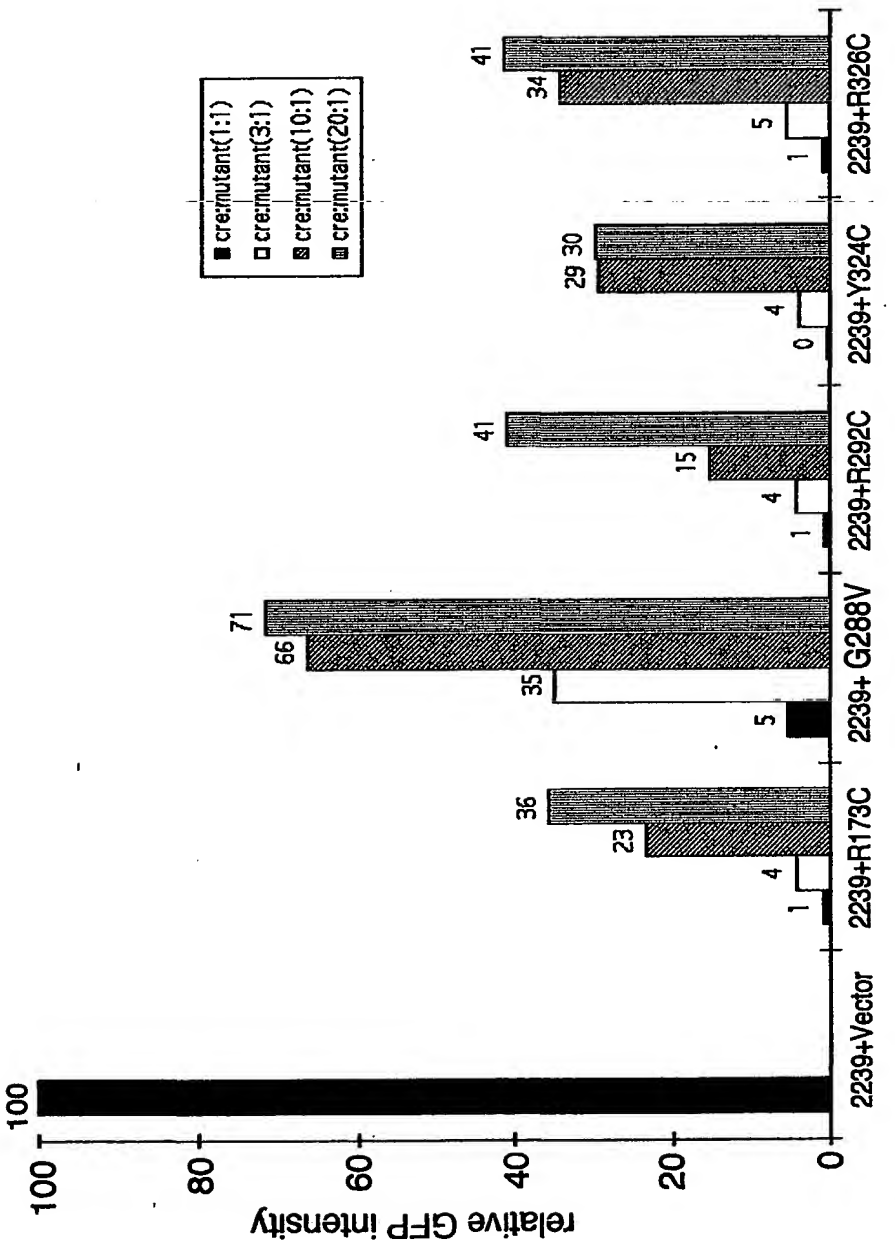


FIG. 10D


relative GFP intensity
100
80
60
40
20
0
cre:mutant(1:1)
cre:mutant(3:1)
cre:mutant(10:1)
cre:mutant(20:1)
2239+Vector
100
2239+R173C
1
4
23
36
2239+ G288V
5
35
66
71
2239+R292C
1
4
15
41
2239+Y324C
0
4
30
29
2239+R326C
1
5
34
41

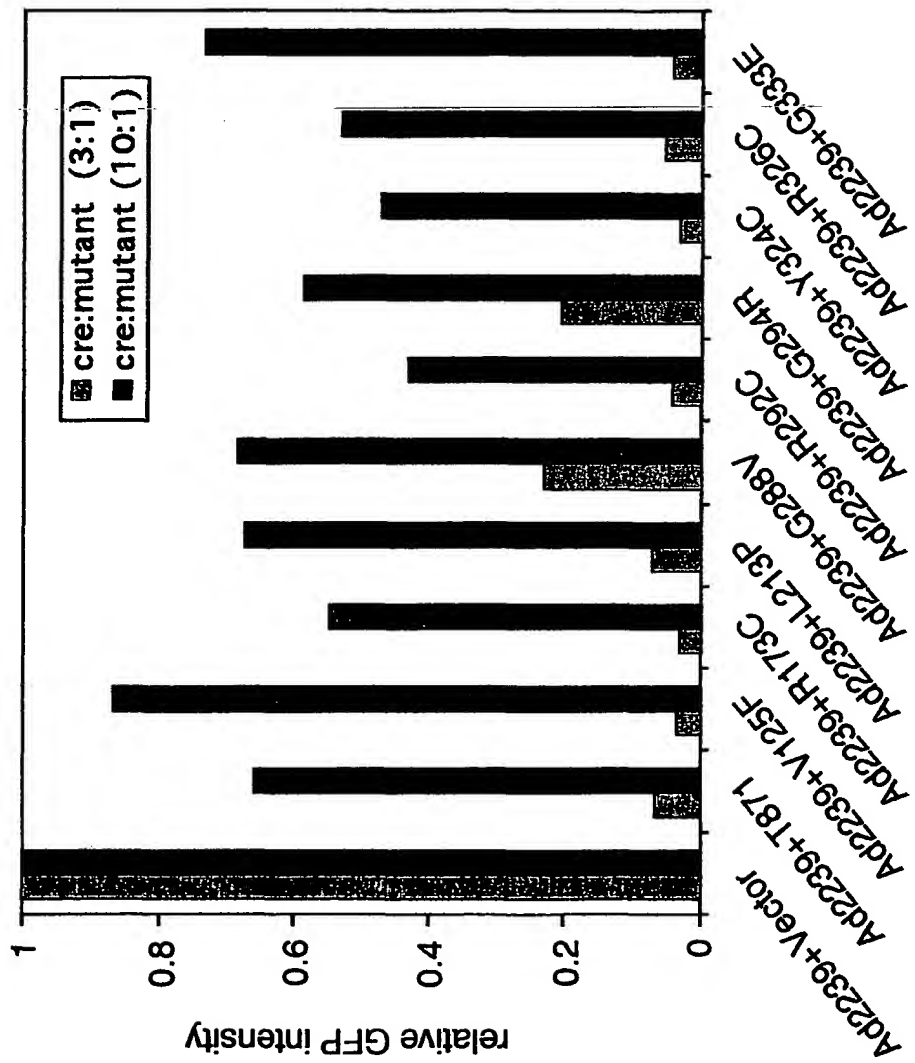

cre:mutant (3:1)
cre:mutant (10:1)
relative GFP intensity
0
0.2
0.4
0.6
0.8
1
Ad2239+Vector
Ad2239+T87I
Ad2239+V125F
Ad2239+R173C
Ad2239+L213P
Ad2239+G288V
Ad2239+R292C
Ad2239+G294R
Ad2239+Y324C
Ad2239+R326C
Ad2239+G333E

FIG. 10E

FIG. 11

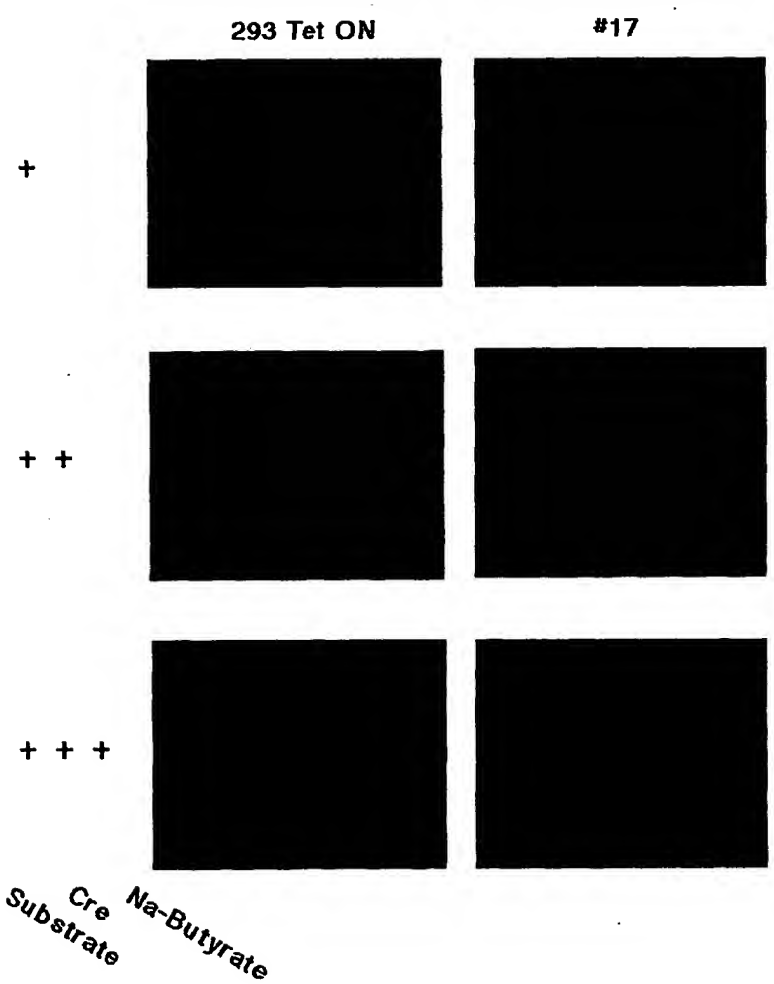


FIG. 12

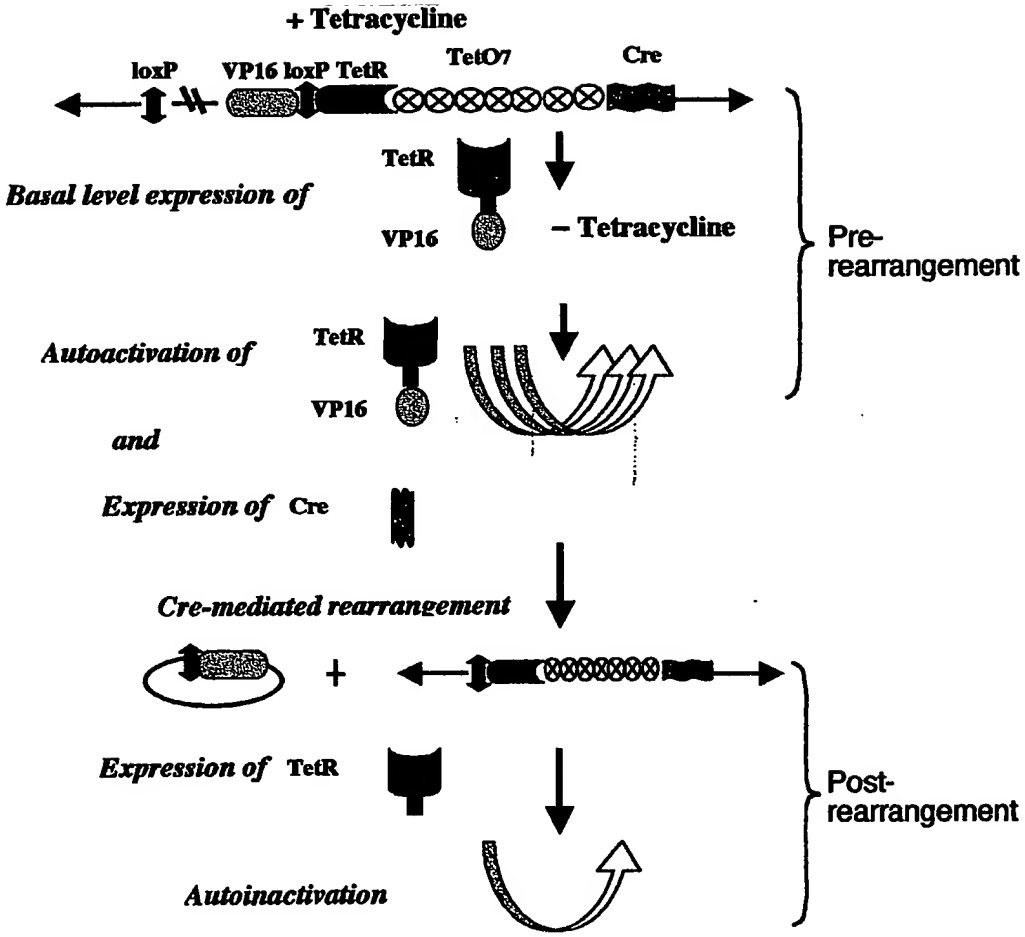
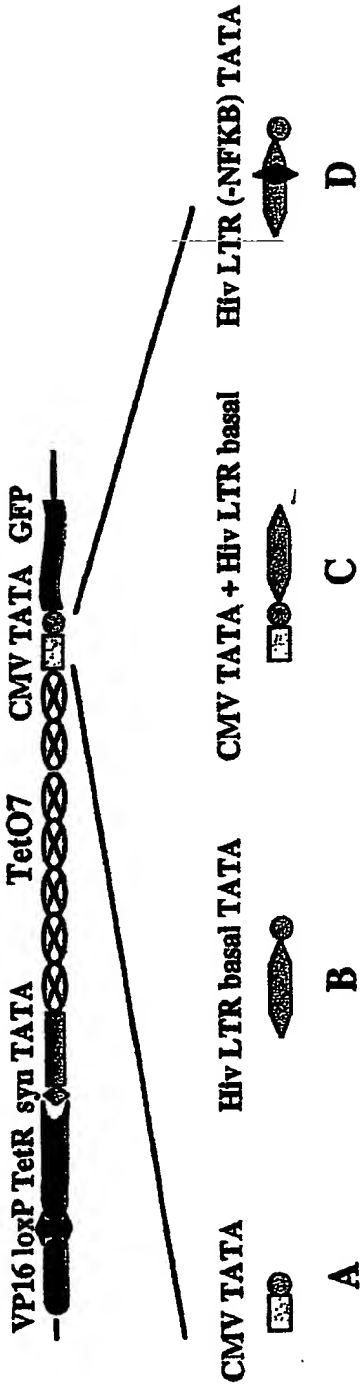

+ Tetracycline
loxP
VP16
loxP
TetR
TetO7
Cre
TetR
VP16
Basal level expression of
– Tetracycline
Pre-rearrangement
Autoactivation of
TetR
VP16
and
Expression of Cre
Cre-mediated rearrangement
+
Expression of TetR
Post-rearrangement
Autoinactivation

FIG. 13A


VP16 loxP TetR syn TATA
TetO7
CMV TATA GFP
A
CMV TATA
Hiv LTR basal TATA
B
CMV TATA + Hiv LTR basal
C
Hiv LTR (-NFKB) TATA
D

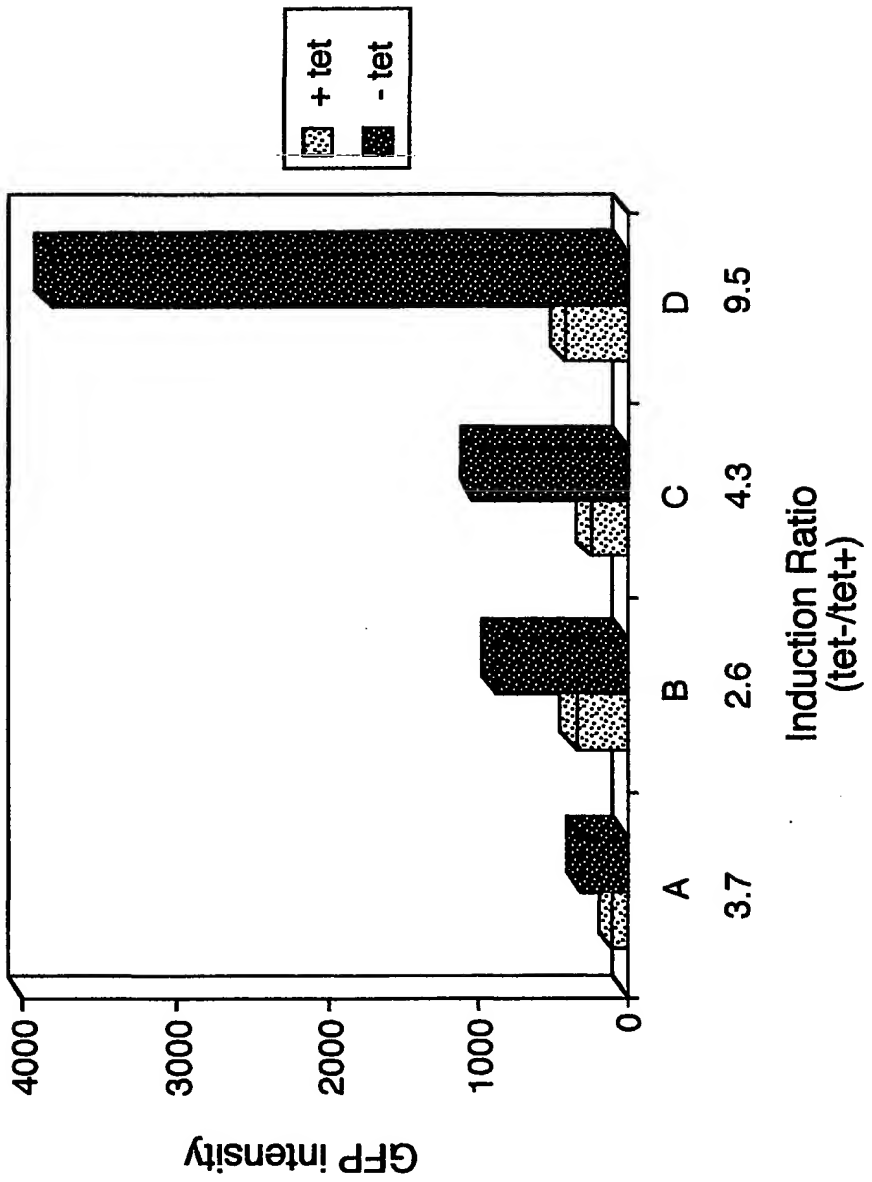

+ tet
- tet
GFP intensity
4000
3000
2000
1000
0
A
B
C
D
3.7
2.6
4.3
9.5
Induction Ratio
(tet-/tet+)

FIG. 13B

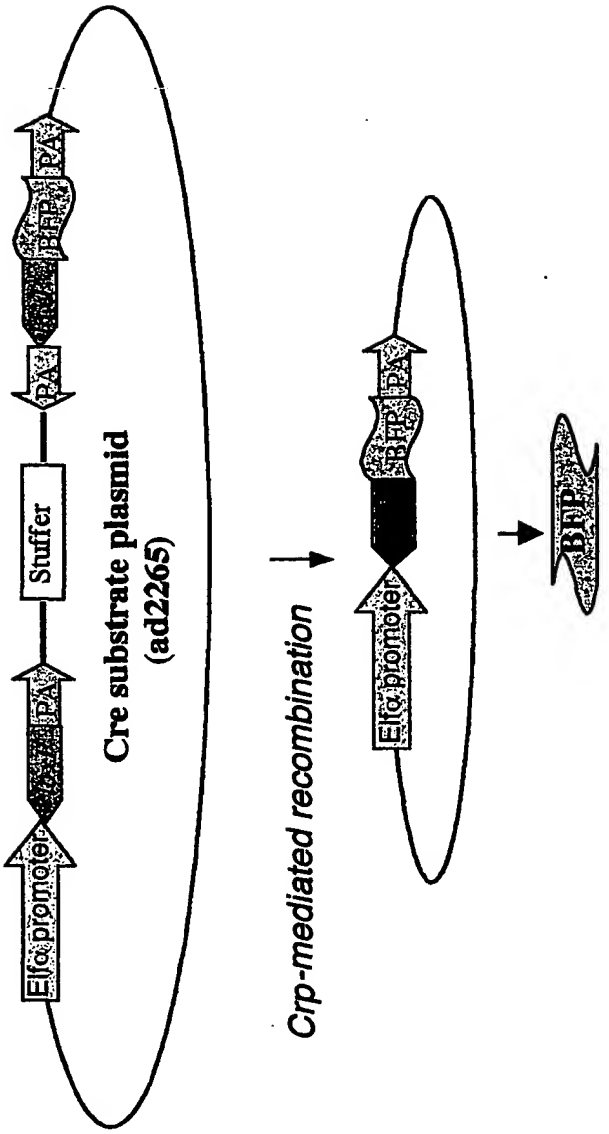

Cre substrate plasmid
(ad2265)
Elfα promoter
PA
Stuffer
PA
BFP
PA
Crp-mediated recombination
Elfα promoter
BFP
PA
BFP

FIG. 14

FIG. 15A

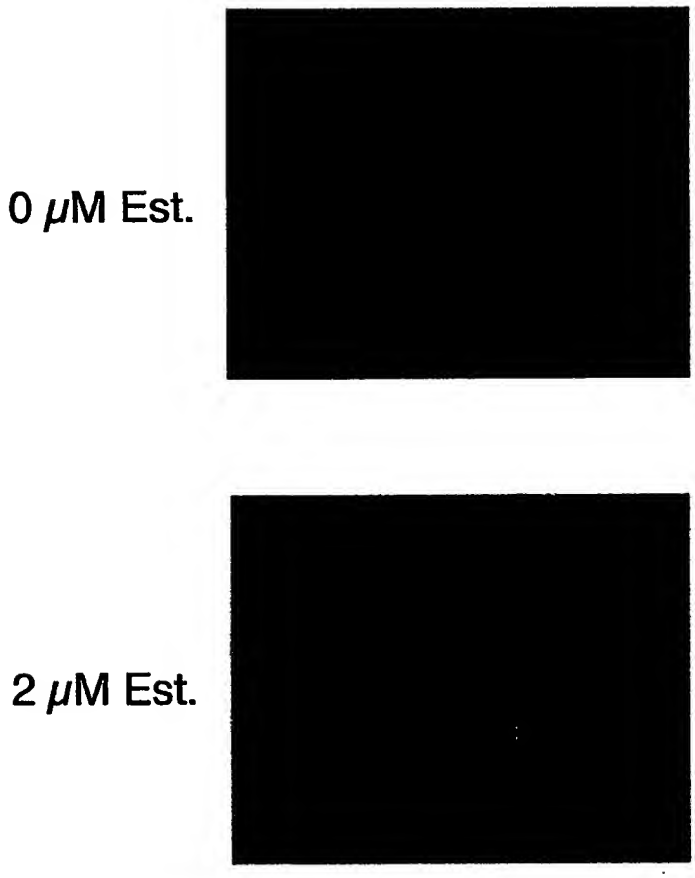


FIG. 15B

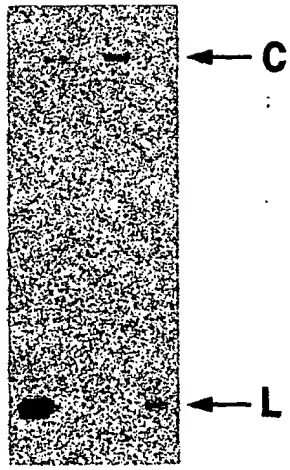


FIG. 16

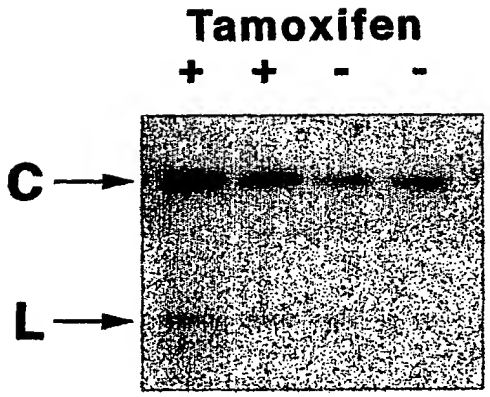


FIG. 17

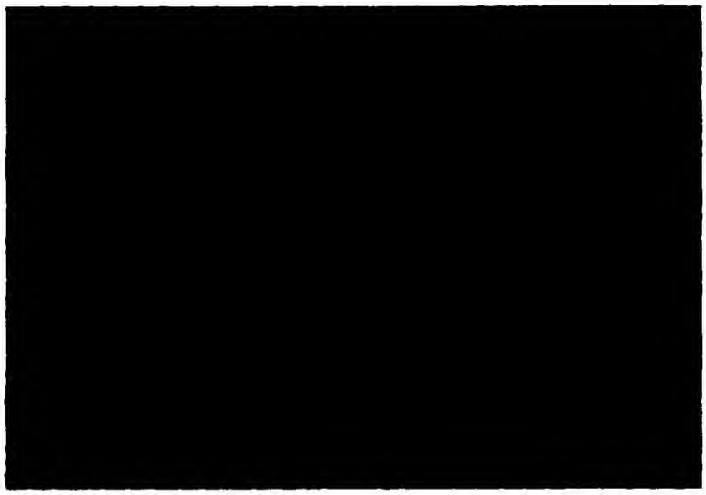
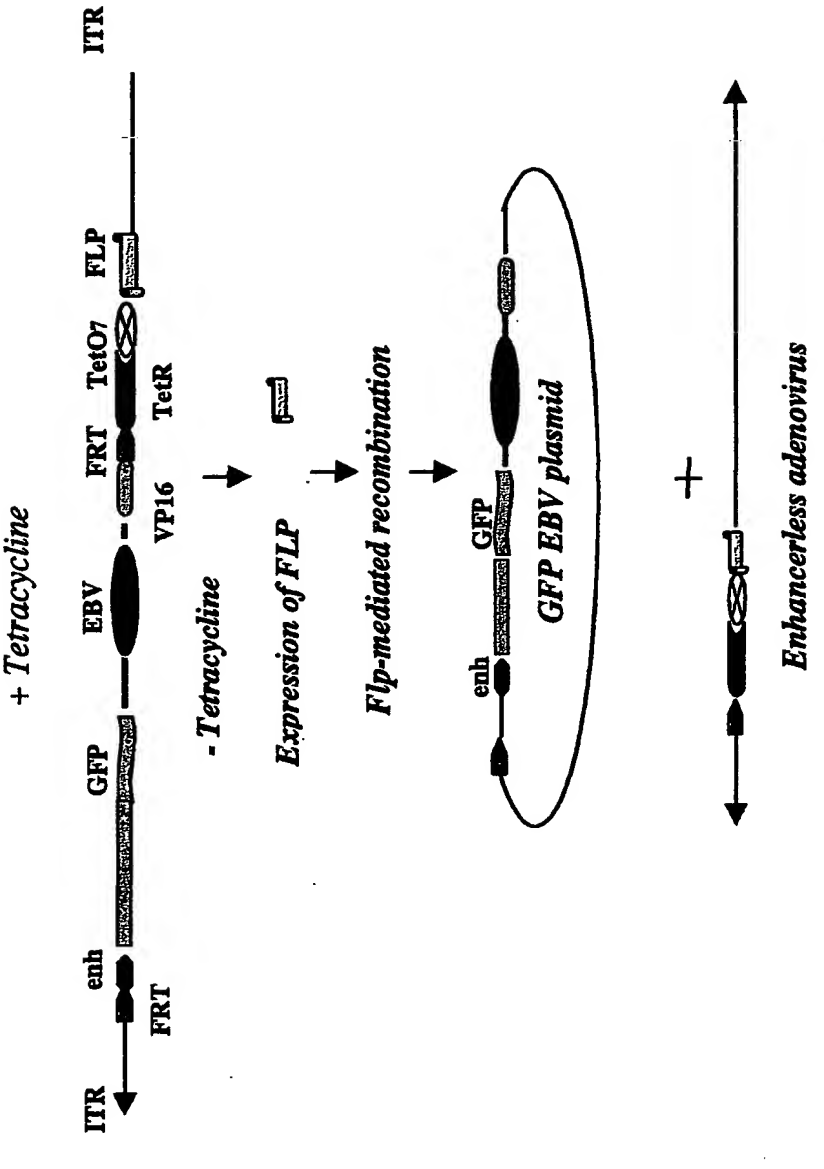


FIG. 18A


ITR
enh
FRT
GFP
EBV
VP16
FRT
TetO7
TetR
FLP
ITR
+ Tetracycline
- Tetracycline
Expression of FLP
Flp-mediated recombination
enh
GFP
GFP EBV plasmid
+
Enhancerless adenovirus

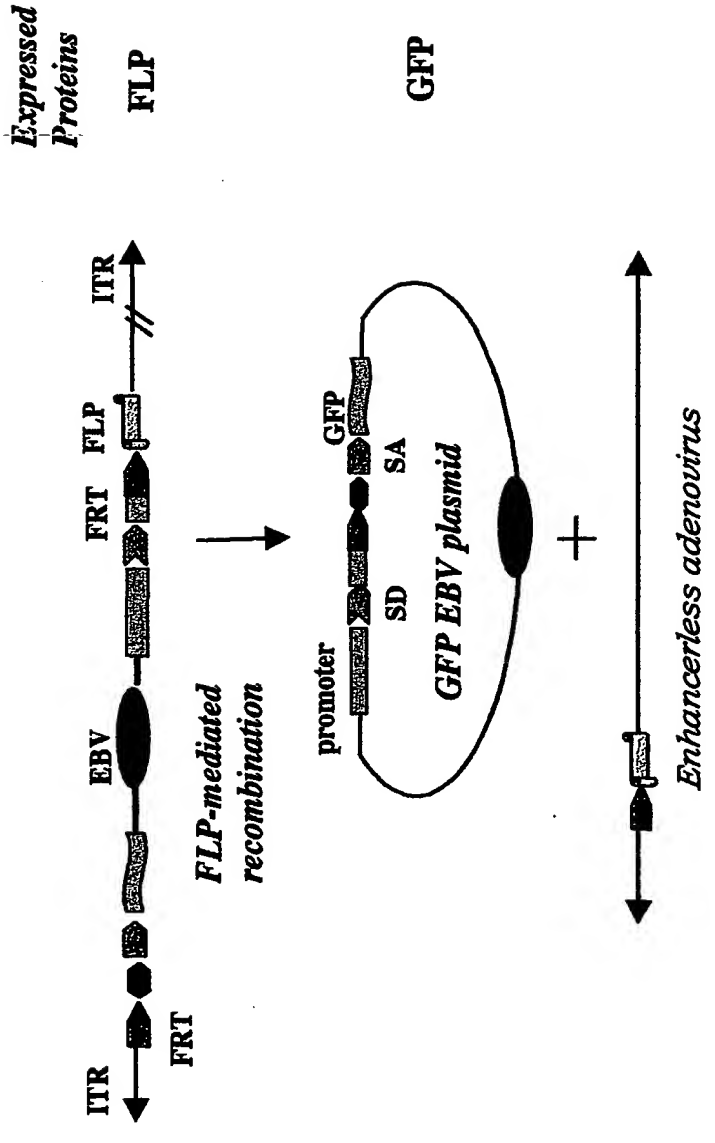

Expressed Proteins
FLP
GFP
ITR
FRT
FLP
EBV
FRT
ITR
FLP-mediated recombination
promoter
SD
SA
GFP
GFP EBV plasmid
Enhancerless adenovirus

FIG. 18B

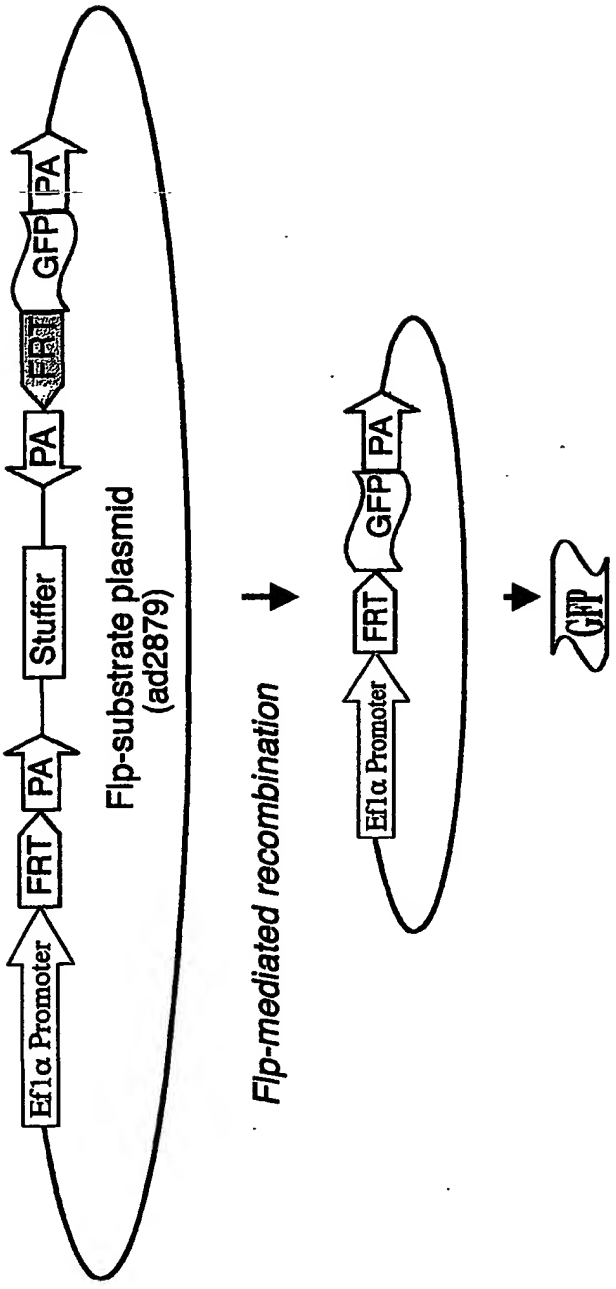

Ef1α Promoter
FRT
PA
Stuffer
PA
FRT
GFP
PA
Flp-substrate plasmid
(ad2879)
Flp-mediated recombination
Ef1α Promoter
FRT
GFP
PA
GFP

FIG. 19A

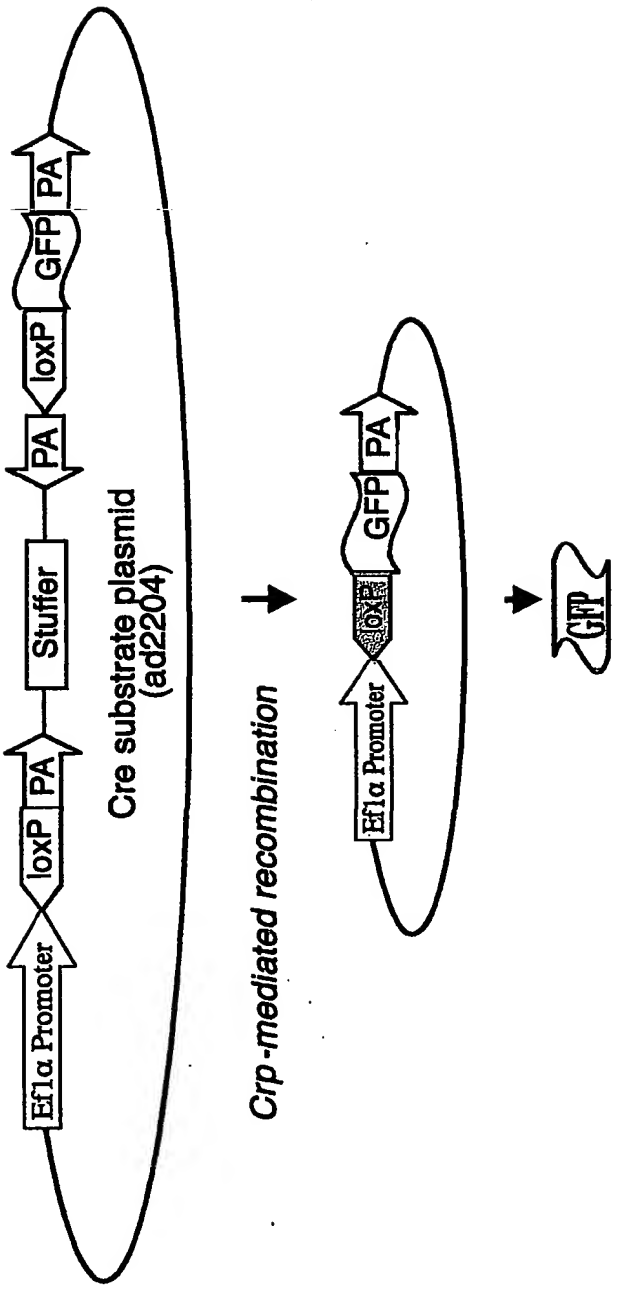

Ef1α Promoter
loxP
PA
Stuffer
PA
loxP
GFP
PA
Cre substrate plasmid
(ad2204)
Crp-mediated recombination
Ef1α Promoter
loxP
GFP
PA
GFP

FIG. 19B

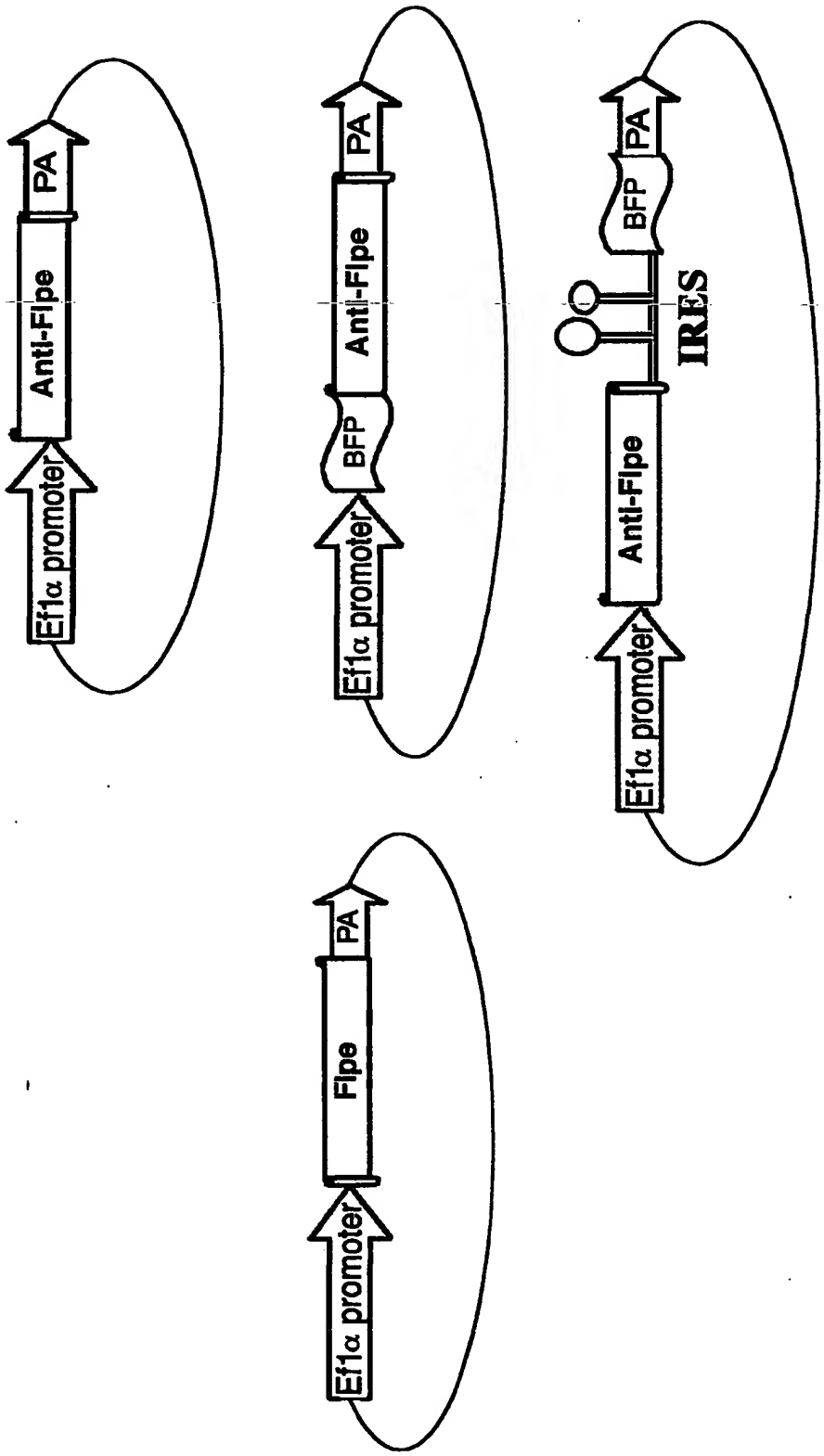
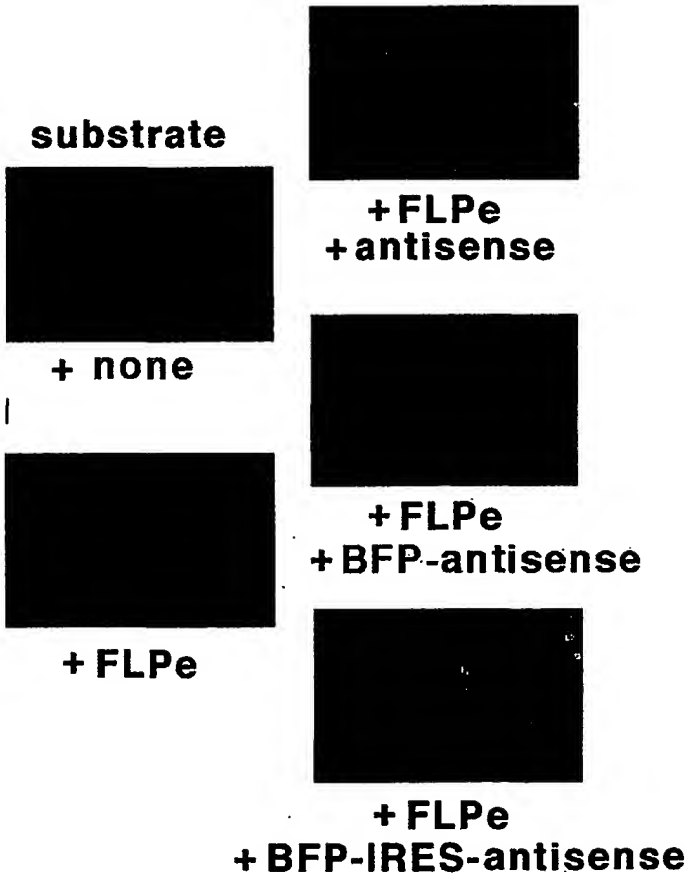

Ef1α promoter
Anti-Flpe
PA
Ef1α promoter
BFP
Anti-Flpe
PA
Ef1α promoter
Anti-Flpe
IRES
BFP
PA
Ef1α promoter
Flpe
PA

FIG. 20

FIG. 21


substrate
+ none
+FLPe
+FLPe
+antisense
+FLPe
+BFP-antisense
+FLPe
+BFP-IRES-antisense

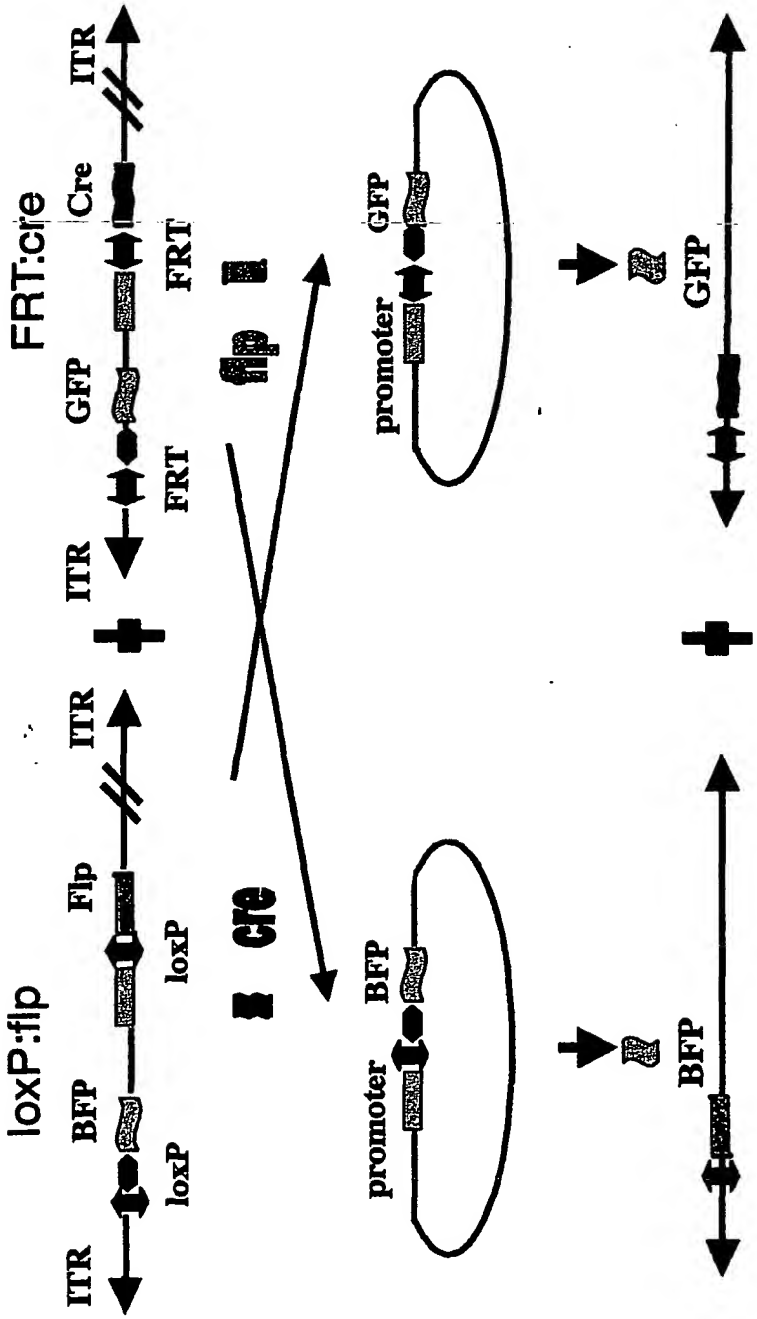

loxP:flp
ITR
BFP
loxP
Flp
loxP
ITR
FRT:cre
ITR
Cre
FRT
GFP
FRT
ITR
flp
cre
promoter
BFP
promoter
GFP
BFP
GFP

FIG. 22

SEQUENCE LISTING

<110> The General Hospital Corporation

<120> Self-rearranging DNA vectors

<130> 00786/352WO3

<150> US 60/231,053
<151> 2000-09-08

<150> US 60/246,904
<151> 2000-11-08

<160> 10

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 2341
<212> DNA
<213> Artificial Sequence

<220>
<223> derived from Adenovirus

<400> 1
gaatcggcca gcgcgaattc gattatcatc atcataatat accttatttt ggattgaagc 60
caatatgata atgaggggtt ggagtttgtg acgtggcgcg gggcgtggga acggggcggg 120
tgacgtaggt tttagggcgg agtaacttgc atgtattggg aattgtagtt tttttaaaat 180
gggaagttac gtacgcggca tcgatgcgcg ggatatcgcg gcggctagcg acatgaggtt 240
gccccgtatt cagtgtcgct gatttgtatt gtctgaagtt gtttttacgt taagttgatg 300
cagatcaatt aatacgatac ctgcgtcata attgattatt tgacgtggtt tgatggcctc 360
cacgcacgtt gtgatatgta gatgataatc attatcactt tacgggtcct ttccggtgat 420
ccgacaggtt acggggcggc gacctcgcgg gttttcgcta tttatgaaaa ttttccggtt 480
taaggcgttt ccgttcttct tcgtcataac ttaatgtttt tatttaaaat accctctgaa 540
aagaaaggaa acgacaggtg ctgaaagcga ggctttttgg cctctgtcgt ttcctttctc 600
tgtttttgtc cgtggaatga acaatggaag ttaacggatc caggccgcga gcaaaaggcc 660
agcaaaaggc caggaaccgt aaaaaggccg cgttgctggc gtttttccat aggctccgcc 720
cccctgacga gcatcacaaa aatcaacgct caagtcagag gtggcgaaac ccgacaggac 780
tataaagata ccaggcgttt ccccctggaa gctccctcgt gcgctctcct gttccgaccc 840
tgccgcttac cggatacctg tccgcctttc tcccttcggg aagcgtggcg ctttctcata 900
gctcacgctg taggtatctc agttcggtgt aggtcgttcg ctccaagctg ggctgtgtgc 960
acgaaccccc cgttcagccc gaccgctgcg ccttatccgg taactatcgt cttgagtcca 1020
acccggtaag acacgactta tcgccactgg cagcagccac tggtaacagg attagcagag 1080
cgaggtatgt aggcggtgct acagagttct tgaagtggtg gcctaactac ggctacacta 1140
gaagaacagt atttggtatc tgcgctctgc caaagccagt taccttcgga aaaagagttg 1200
gtagctcttg atccggcaaa caaaccaccg ctggtagcgg tggtttttt gtttgcaagc 1260
agcagattac gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt 1320
ctgacgctca gtggaacgaa aactcacgtt aagggatttt ggtcatcaga ttatcaaaaa 1380
ggatcttcac ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taaagtatat 1440
atgagtaaac ttggtctgac agttaccaat gcttaatcag tgaggcacct atctcagcga 1500
tctgtctatt tcgttcatcc atagttgcct gactccccgt agtgtagata actacgatac 1560
gggagggctt accatccggc cccagtgctg caatgatacc gcgtgaccca cgctcaccgg 1620
ctcctgattt atcagcaata aaccagccag ccggaagtgc cgagcgcaga agtggtcctg 1680
caactttatc cgcctccatc cagtctatta gttgttgccg ggaagctaga gtaagtagtt 1740

cgccagttaa tagttttcgc aacgttgttg ccattgctac aggcatcgtg gtgtcacgct 1800
cgtcgtttgg tatggcttca ttcagctccg gttcccaacg atcaaggcga gttacatgat 1860
cccccatgtt gtgcaaaaaa gcggttagct ccttcggtcc tccgatagtt gtcagaagta 1920
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/27682

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/86, 15/861, 5/10, 15/11, 15/63, 15/64, 15/65; A61K 48/00

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.2, 93.6; 435/320.1, 69.1, 455, 456, 457, 325, 369, 91.1, 91.4, 91.42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	US 6,080,576 A (ZAMBROWICZ et al.) 27 June 2000 (27.06.00), see entire document, especially Figure 2, claims 1-3 and columns 10 and 16.	1-2, 4-11, 13
A	US 5,919,676 A (GRAHAM et al.) 06 July 1999 (06.07.99), see entire document, particularly claims 1-6 and columns 2 and 5-6.	1-34
A	AGAH et al. Gene recombination in postmitotic cells. Journal of Clinical Investigation. July 1997, Vol. 100, No. 1, pages 169-179, especially pages 171-173.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priorit date and not in conflict with the application but cited to understan the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot b considered novel or cannot be considered to involve an inventive ste when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot b considered to involve an inventive step when the document is combin with one or more other such documents, such combination bei obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 01 NOVEMBER 2001	Date of mailing of the international search report 31 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer David Guzo Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/27682

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.1, 93.2, 93.6; 435/320.1, 69.1, 455, 456, 457, 325, 369, 91.1, 91.4, 91.42

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, Dialog, NTIS, Medline, Biotech, Biosis, Biosci, Chemical Abstracts
Search terms: adenovirus, recombinase, target site, Cre, cleavage site, gene therapy

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